Effects of Lifestyle and Environment on Human Sperm

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

3-DG	3-deoxyglucosone
5MedCyd	5-methyl-cytosine
ADAM2	Fertilin Subunit Beta
AGE	Advanced glycation end products
ART	Assisted reproductive techniques
AS	Angelman syndrome
ATP	Adenosine triphosphate
BMI	Body mass index
bp	Base pair
BPA	Bisphenol A
BSA	Bovine serum albumin
BWS	Beckwith-Weidemann Syndrome
CCF	Corrected total cell fluorescence
CEL	Carboxyethyllysine
CML	Carboxymethyllysine
COMET	Single Cell Gel Electrophoresis
CpG	Cytosine-Guanine site
CSSB	Caffeinated sugar sweetened beverages
CTCF	CCCCTC-binding factor
DAZL	Deleted in Azoospermia Like
DDT	dichlorodiphenyltrichloroethane
DLK1	Delta-Like 1 Homolog (Drosophila)
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DV	Dependent variable
ECM	Extracellular matrix
EDC	Endocrine Disrupting Chemical
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle Stimulating Hormone
GO	Glyoxal
GOLD	Glyoxal lysine dimer
GPI	Glycosyl phosphatidylinositol
GST	Glutathione-S-transferase
H19	Non protein coding
HA	Hyaluronic acid
HAS	Human serum albumin
HBA	Hyaluronic acid binding assay

HbA_{1c}	Glycated haemoglobin
HEPES	4-(2-hydroxyethyl)1-piperazineethanesulfonic acid
HPG	Hypothalamic-Pituitary-Gonadal
ICC	Immunocytochemistry
ICR	Imprinting control region
ICSI	Intracytoplasmic sperm injection
IG	Intergenic
IGF2	Insulin-like Growth Factor 2
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IV	Independent variable
IVF	In vitro fertilisation
IZUMO1	Izumo Sperm-Egg Fusion 1
Kb	Kilobase
LH	Luteinising Hormone
LINE-1	Long Interspersed Element 1
LSI	Leisure score index
MEG3	Maternally Expressed Gene 3
MEST	Mesoderm specific transcript
MFI	Male factor infertility
MFi	Mean fluorescence intensity
MG	Methylglyoxal
MOLD	Methylglyoxal lysine dimer
mRNA	Messenger ribonucleic acid
MTHFR	Methylenetetrahydrofolate reductase
NaCl	Sodium chloride
NFkB	Nuclear-factor kappa beta
NR3C1	Nuclea Receptor Subfamily 3, Group C, Member 1
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PLAGL1	Pleiomorphic Adenoma Gene Like 1
PM	Progressive motility
PVC	Polyvinylchloride
PWS	Prader-Willi syndrome
Q-Q	Quantile-quantile
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAM	S-adenosylmethionine
SCSA	Sperm Chromatin Structure Assay
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SGP	Slow growth period
SNP	Single nucleotide polymorphism
SNPRN	Small Nuclear Ribonucleoprotein N

SPAM1	Sperm adhesion molecule 1
SPM	Sperm preparation media
SRS	Silver-Russel syndrome
SSB	Sugar sweetened beverages
TBE	Tris buffered saline
TUNEL	Terminal Deoxynucleotidyl Transferase-mediated Nick End Labeling
UV	Ultraviolet
WBC	White blood cell
WHO	World Health Organisation
ZP	Zona pellucida

Abstract

There is considerable evidence to suggest that male fertility is decreasing worldwide, and it is thought that lifestyle and environmental factors could play a role. Lifestyle and environmental information was collected from men attending a fertility clinic by questionnaire and correlated with sperm parameters and the outcomes of assisted reproductive techniques (ART). Global and gene-specific DNA methylation in these men was measured by bisulphite pyrosequencing and compared with questionnaire results. Finally, an *in vitro* glycation assay was developed to investigate the effects of advanced glycation end products (AGEs) on sperm: AGE formation, oxidative DNA damage and reactive oxygen species (ROS) production were then measured using flow cytometry and immunocytochemical methods.

BMI was the main determining factor for sperm quality and ART outcomes in this study: high BMI was associated with lower sperm concentration and lower fertilisation rates in *in vitro* cytoplasmic sperm injection (ICSI) patients. Surprisingly, having an unhealthy diet was positively associated with progressive sperm motility. DNA methylation analysis revealed that exposure to glues, adhesives and resins was associated with higher global DNA methylation. A major marker of low sperm concentration was promoter hypermethylation of the spermatogenesis-associated gene, *DAZL*. Furthermore, global DNA methylation and *MEG3* methylation were higher in men with greater numbers of immotile sperm and those with low sperm motility, respectively. Overall, elevated DNA methylation was found to be the main feature of poor sperm quality, however prepared sperm showed higher methylation than whole sperm from neat semen. This large study indicates that DNA methylation is likely to be linked to sperm function, although the role of lifestyle-acquired epimutations in this may be minor.

Treatment of sperm with glyoxal *in vitro* caused rapid formation of the major AGE-adduct, CML. This occurred alongside an increase in oxidative DNA damage but did not affect sperm hyaluronidase activity. This study shows that one of the mechanisms of AGE-related DNA damage is due to oxidative stress and this may present a risk to sperm DNA integrity *in vivo.*

Chapter 1

Introduction

1.1 The male gamete

1.1.1 Sperm cell structure and function

The sperm cell is the physiological carrier of the haploid paternal deoxyribonucleic acid (DNA) to the oocyte containing the haploid maternal DNA whereupon fertilisation occurs and the fusion of the parental genomes gives rise to a developing embryo. The sperm cell is a highly specialised motile cell, containing features that enable optimal transit from the male reproductive tract, through the female reproductive tract and to the oocyte with the paternal genome protected throughout.

The sperm cell is 60-70 µm in length and made up of three major sections: the head, the midpiece and the tail (fig. 1.1) (Jones & Lopez, 2013). The sperm head contains the nucleus which carries the tightly compacted DNA and, unlike somatic cells, contains very little cytoplasm. At the anterior position of the head is the acrosome, a specialised secretory vesicle that contains digestive enzymes exocytosed upon sperm-egg binding during the acrosome reaction to break down the structural components of the zona pellucida surrounding the oocyte (Harper *et al.,* 2008). The midpiece section contains large numbers of mitochondria which generate adenosine triphosphate (ATP) by oxidative phosphorylation to drive tail movement. The motile tail contains a central axoneme surrounded by two central singlet microtubules and nine doublet microtubules and flagellar movement is caused by the sliding of microtubule doublets past each other by ATP-driven dynein motor proteins (Alberts, Johnson, Lewis, Raff, Roberts, & Walter, 2002). The sperm centriole, located in the sperm neck at the anterior midpiece is the origin of the flagellar axoneme

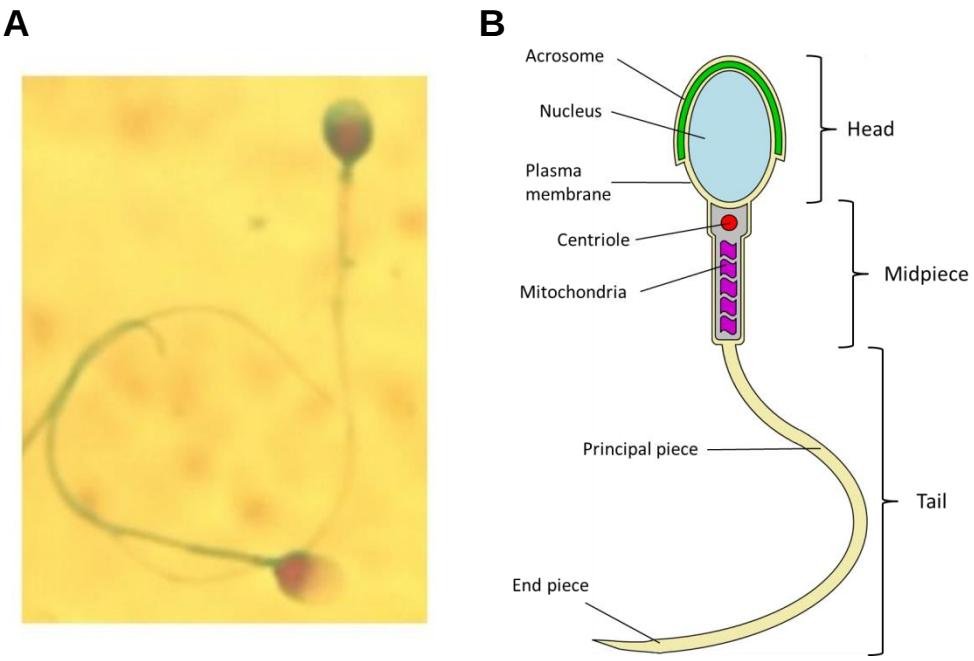


Figure 1.1 Structure of the sperm cell

Morphology of the sperm cell as visualised with a light microscope following staining (A) and a schematic diagram of cellular components (B).

1.1.2 Spermatogenesis

The journey of a sperm cell begins in the testis, specifically within the nbvseminiferous tubules where sperm stem cells, known as spermatogonia, give rise to mature sperm. Spermatogonia are diploid (2n) and divide mitotically to maintain the pool of sperm stem cells. To generate mature sperm, a spermatogonium first divides meiotically to produce primary and then secondary haploid spermatocytes (n). Secondary spermatocytes then undergo a second meiotic division to produce four haploid cells, called spermatids. Spermatids finally differentiate into mature sperm by spermiogenesis, through which spermatids become elongated, the acrosome forms and the nucleus condenses (Zhang et al., 2014; Jones and Lopez, 2013). These events take place adjacent to supporting Sertoli cells which release mature sperm into the lumen of the seminiferous tubules. The time frame for the development of mature sperm from spermatogonia is around

74 days and as this is a continual process, at any given time, cells at all stages are present in the seminiferous epithelium.

1.1.3 Sperm chromatin packaging

During spermatogenesis, sperm DNA undergoes structural and chemical reorganisation in order to form a condensed nucleus in which the DNA is protected during transit to the oocyte, is transcriptionally inactive and which has a reduced volume to aid motility. Unlike in somatic cells in which DNA-bound histones arrange into larger nucleosomes, the major DNA-binding protein in sperm is the protamine (Braun, 2001). During spermatogenesis, histone proteins are replaced firstly by intermediary transition proteins, then by protamines 1 and 2 which allows packaging of the DNA into a volume less than 5% of a somatic cell nucleus (Miller *et al.,* 2010)+. The importance of this process is illustrated by the fact that improper histone replacement causes infertility in mice has also been linked to infertility in men (Cho *et al.,* 2001; Zhang *et al.,* 2006). Other post-translational modifications of histones occur to achieve a compact chromatin state including controlled region-specific acetylation, phosphorylation and methylation (Govin *et al.,* 2007; Meyer-Ficca *et al.,* 2005; Godmann *et al.,* 2007).

1.1.4 Capacitation, the acrosome reaction and fertilisation

After ejaculation and following deposition in the female reproductive tract sperm undergo a number of physiological changes to acquire their fertilising capacity. Capacitation is the process in which the sperm cell becomes hypermotile to enable rapid directional swimming towards the oocyte and undergoes membrane changes that prime the cell for zona pellucida binding and the acrosome reaction. This occurs in the female genital tract.

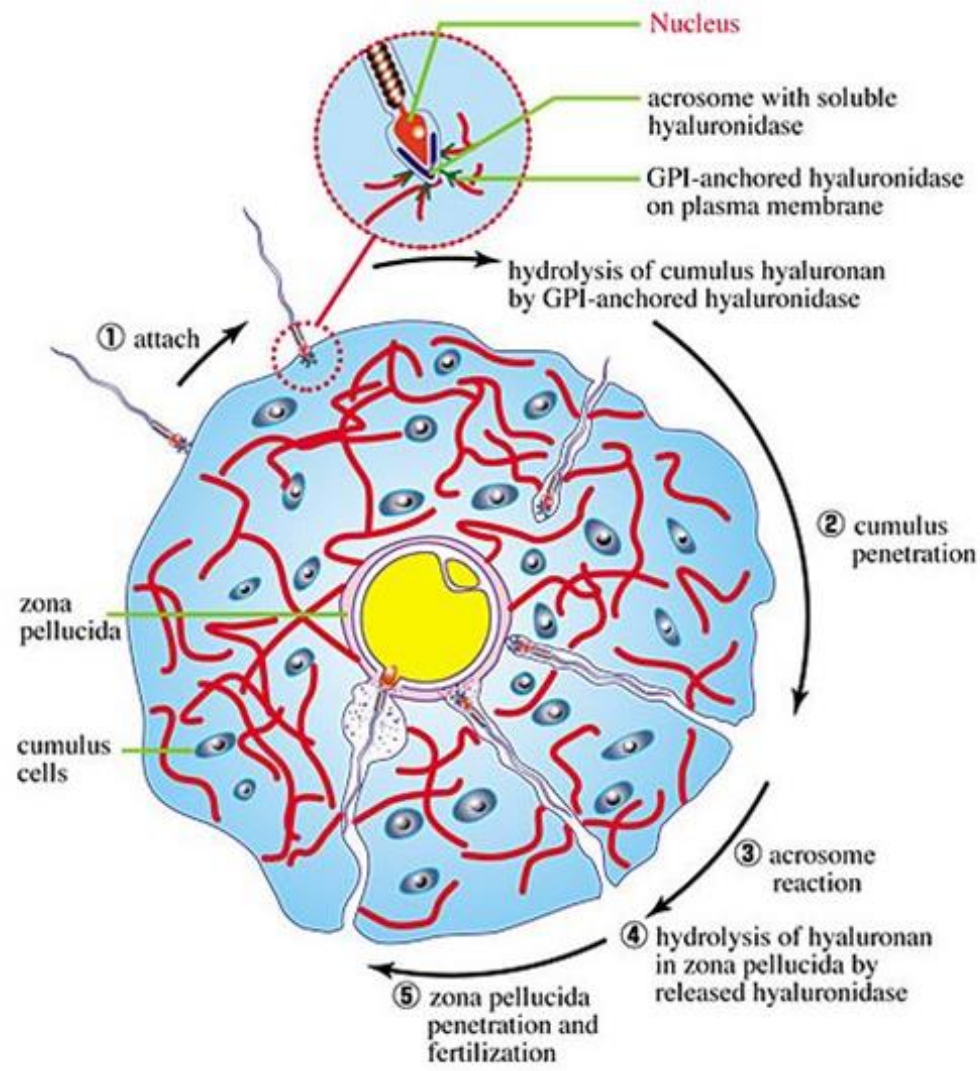


Figure 1.2 Fertilisation

Membrane-bound hyaluronidase degrades HA in the ECM following initial attachment to the cumulus layer (1 and 2). Soluble hyaluronidase is released during the acrosome reaction (3) to degrade HA at the ZP (4), allowing fertilisation (4).

Adapted from (Salustri & Fulop, 1998)

The molecular changes to the cell include increased membrane fluidity which is achieved by modifying the cholesterol content of the membrane and increased permeability to Ca^{2+} ions; these changes are necessary for full capacitation and to prepare sperm for Ca^{2+}signalling which leads to acrosomal exocytosis (Morales *et al.,* 2012; O'Toole *et al.,* 2000). Reactive oxygen species (ROS) production, protein tyrosine phosphorylation and actin polymerisation are other important processes in capacitation (review in

Aitken and Nixon, 2013; Flesch *et al.,* 2001; Lopez-Gonzalez *et al.,* 2014; Itach *et al.,* 2012). These changes are essential as only capacitated sperm can fertilise an oocyte (Quill *et al.,* 2003).

Prior to reaching the ZP, sperm must penetrate the surrounding cumulus layer. This layer contains a large number of loosely packed cumulus cells embedded in a dense extracellular matrix (ECM) of hyaluronic acid (HA) oligosaccharide chains. The sperm hyaluronidase Sperm Adhesion Molecule 1 (SPAM1/PH-20) is a membrane glycosyl phosphatidylinositol-(GPI)-linked enzyme that is responsible for the initial degradation of HA in the cumulus ECM at a neutral pH (Lin *et al.,* 1994) (fig 2.2). Sperm hypermotility, activated during capacitation, facilitates forceful burrowing through the cumulus layer where sperm then can bind to the ZP. The ZP is an ECM made up of three glycoproteins: ZP1, ZP2 and ZP3, to which as yet unclassified receptors on the sperm membrane bind (reviewed in Chiu *et al.,* 2014a). This binding triggers the acrosome reaction in sperm, in which the membrane of the acrosomal vesicle fuses with the sperm plasma membrane and its contents is exocytosed. The enzymes released allow degradation of the ZP and include a soluble form of SPAM1 with acidic pH, which allows further cleavage of HA in the ZP (Reese *et al.*, 2010) (fig. 2.2).

The paternal DNA is deposited into the ovum upon fusion of the sperm and ovum plasma membranes whereupon the paternal DNA is decondensed and the paternal and maternal pronuclei merge and the zygote is formed.

1.1.5 Reactive oxygen species and oxidative stress

Reactive oxygen species (ROS) such as the superoxide anion ($\cdot O_2^-$), hydroxyl radicals ($\cdot OH$) and hydrogen peroxide (H_2O_2) are produced during normal aerobic metabolism in all cells including sperm. ROS are essential for normal sperm function, including for capacitation, hyperactive motility, and sperm maturation (de Lamirande and Gagnon, 1993; Gil-Guzman *et al.,* 2001). Despite the beneficial actions of ROS, their levels must be controlled to prevent toxicity, particularly against free radicals which are highly reactive

due to their unpaired electrons which allow them to oxidise lipids, amino acids and carbohydrates. Sperm are protected from the effects of these free radicals by the action of radical scavenging enzymes present in sperm and in seminal plasma. These enzymes include superoxide dismutase, catalase and glutathione peroxidase, which provide antioxidant protection to the sperm (Zini *et al.,* 2002; Giannattasio *et al.,* 2002; Lewis *et al.,* 1995; Noblanc *et al.,* 2011). However, when ROS levels exceed the cell's antioxidant capacity, oxidative stress occurs, to which sperm are particular susceptible due to the high levels of polyunsaturated fatty acids in the plasma membrane (reviewed in Aitken, Jones and Robertson, 2012). One of the most damaging consequences of oxidative stress is that is causes DNA damage, and this has been identified as a characteristic of sperm from infertile men (Mahfouz *et al.,* 2010; Agarwal *et al.,* 2006). Furthermore, DNA damage in the male germ line has been linked to adverse outcomes of assisted reproductive techniques (ART), particularly in patients undergoing *in vitro* cytoplasmic injection (ICSI) (Simon *et al.,* 2013; Loft *et al.,* 2003; Zini *et al.,* 2011), therefore this is a critical risk factor for disrupted development. Animal studies have shown that exposure to compounds that induce oxidative stress can cause miscarriage or morbidity in the offspring.

1.2 Male factor infertility

The global prevalence of infertility is reported to be approximately 15% with males accounting for 20-30% of infertile cases (European Society of Human Reproduction and Embryology, 2014). Causative factors of male factor infertility (MFI) impair sperm function and integrity, affecting sperm motility, morphology and vitality (Guzick et al., 2001) and a number of studies have reported decreasing male fertility in recent years (Carlsen *et al.,* 1992; Auger *et al.,* 1995; Rolland *et al.,* 2013; Romero-Otero *et al.,* 2015; Centola *et al.,* 2016). Male factor infertility can be acquired, as in testicular injury, sexually transmitted disease and cancer (Rowe, 2000); or it can arise from congenital problems, including endocrinopathies such as androgen insensitivity

syndrome (Pitteloud and Dwyer, 2014), developmental disorders such as cryptorchidism (Lee *et al*., 1997), and chromosomal abnormalities such as Klinefelter syndrome or Y chromosome microdelections (Stouffs *et al*., 2014). Sperm production and quality can also be influenced by aspects of lifestyle, such as diet and exercise, and environmental exposures to toxicants, or other xenobiotics (reviewed by Sharpe, 2010). As the most common type of male infertility is idiopathic – exhibiting one or more abnormal semen parameters with no identifiable cause (Baker, 1994) – there is increasing interest in the impact of these latter factors, and the epigenetic mechanisms underlying them. The changes to semen parameters associated with MFI include reduced concentration (oligozoospermia) or a complete absence of sperm (azoospermia), poor sperm motility (asthenozoospermia), abnormal sperm morphology, or significantly reduced seminal volume. The World Health Organisation (WHO) has classified threshold values of semen parameters for male infertility and these are as follows: sperm concentration, $15x10^6$/ml; total motility, 40%; progressive motility, <32%, total sperm count, $39x10^6$; morphology, 4% normal forms; and semen volume, <1.5 ml (Cooper *et al.,* 2010).

1.2.1 Assisted reproductive technologies

As the levels of infertility have risen, so has the use of ART due to the increased availability of treatment in most countries (Inhorn and Patrizio, 2015). The number of children born through ART now stands at over 5 million (European Society of Human Reproduction and Embryology, 2014), which highlights the importance of this as a standardised therapy for infertility. Upon consultation at a fertility clinic, semen parameters are analysed to determine if the patient has MFI according to the criteria described above. In cases where these parameters are normal, *in vitro* fertilisation (IVF) is usually carried out, in which sperm and egg are incubated together and sperm must bind and penetrate the ZP and fuse with the egg plasma membrane as described earlier. IVF treatment is mostly used for female factor infertility. For cases with MFI, the primary treatment is ICSI

whereupon a single sperm is selected by an observer and injected directly into the oocyte. In this scenario, sperm bypass the normal cellular and molecular barriers to fertilisation and the paternal DNA is delivered directly. ICSI is in fact the primary technology used, comprising approximately two thirds of all fertility treatments worldwide (European Society of Human Reproduction and Embryology, 2014).

1.2.2 Lifestyle and environmental factors associated with MFI

The increase in prevalence of MFI is thought to be linked to several environmental and lifestyle factors which are affecting sperm parameters. A major concern is that many of the damaging consequences of these lifestyle factors may manifest at the molecular and DNA level, in which case they will not be identified by standard semen analysis upon consultation for ART.

1.2.2.1 Obesity

Overweight and obesity are the most important predictors of diabetes, and the current obesity epidemic is due to a combination of genetic, epigenetic and environmental factors including diet and sedentary lifestyles (Hu *et al*., 2001; Chen *et al*., 2012; Locke *et al*., 2015). The rapid rise in obesity has occurred alongside the reported decreases in semen quality and many studies have investigated the effects of this on MFI. A 2010 meta-analysis by McDonald *et al.* involving 31 studies found no evidence of an association between body mass index (BMI) and sperm concentration or total sperm count, although there were differences in testosterone and sex-hormone binding globulin. However, more recent studies have suggested that there is a role for obesity in reducing sperm quality (Sermondade *et al.,* 2013; Eisenberg *et al.,* 2014). Furthermore, obesity is associated with a longer time to pregnancy in the general population (Ramlau-Hansen *et al.,* 2007a). The deregulation of male reproductive hormones is one of the main hypotheses behind reproductive dysfunction in obese men. Al-Ali *et al.* (2014) found that BMI was not related to sperm quality, based on concentration, motility and morphology, but they did find significant changes in male hormone levels,

luteinising hormone (LH) and testosterone. In males, oestrogens exert a negative feedback effect on the hypothalamic-pituitary-gonadal (HPG) system causing a decrease in the production of gonadotrophins (LH and follicle stimulating hormone (FSH)) and there is a strong hypothesis that obesity deregulates this HPG axis. Furthermore, although there may not be changes in standard semen parameters, numerous studies have shown increases in DNA damage in the sperm of obese men attending fertility clinics (Chavarro *et al.,* 2010; Dupont *et al.,* 2013), although a recent study of 1503 men from the general population found no association between sperm DNA damage and BMI (Bandel *et al.,* 2015). Nevertheless, this DNA damage is thought to be driven by obesity-induced ROS production leading to oxidative stress (Tunc *et al.*, 2011) and as ROS levels are already a marker for infertility, the effects of obesity on sperm may be more pronounced in men with existing fertility problems.

1.2.2.2 Smoking

Tobacco smoke is known to be hugely detrimental to many aspects of health and yet smoking prevalence remains staggeringly high with no clear sign of reducing (Ng, *et al.,* 2014). Many publications have reported adverse effects of smoking on sperm parameters (Künzle *et al.,* 2003; Zhang *et al.,* 2013; Hamad *et al.,* 2014), which is thought to occur in a dose-dependent manner (Ramlau-Hansen *et al.*, 2007b) and to improve upon smoking cessation (Santos *et al.,* 2011). Similarly to the effects of obesity, smoking has been shown to be associated with altered reproductive hormone levels through altering the HPG axis (Jeng *et al.,* 2014; Ramlau-Hansen *et al.,* 2007b), which could be a source of deregulated spermatogenesis leading to poorer sperm quality. However, hormone levels were not found to be different in the sperm of fertile smokers (Pasqualotto *et al.,* 2006). Alternatively, or in conjunction with this effect, the generation of oxidative stress through smoking could be a source of damage to sperm (Saleh *et al.,* 2002). As smoking has been linked to abnormal histone to protamine transition (Hamad *et al.,* 2014), this disrupted chromatin state could lead to a vulnerability of sperm DNA to oxidative stress. Furthermore, smoking has been found to be

associated with aberrant sperm parameters in fertile men, highlighting the wide-ranging effect of this habit (Vine, 1996). Higher levels of sperm DNA damage could explain the poorer pregnancy outcomes that have been associated with paternal smoking (Joesbury *et al.,* 1998; Venners, 2004), however the evidence for this is limited as smokers are excluded from having ART treatment and therefore data on this is not available.

1.2.2.3 Environmental toxicants

The damaging health implications of environmental toxicants are well documented and there is increasing concern over whether chemical exposure in industrialised countries, which is becoming more common, is affecting male reproductive function. Cross-sectional studies on chemical exposure have been the main source of information on the impacts on sperm. For example, Rubes *et al.* (2005) found an increase in sperm DNA fragmentation in men with higher exposure to air pollution, and importantly this occurred without a change in standard semen parameters. Furthermore, a study of men exposed to traffic pollutants through working at motorway tollgates found a decrease in total motility and forward progression in their sperm (De Rosa *et al.,* 2003). As air quality standards in urban environments remain low (World Health Organisation, 2014), there is cause for concern for male reproductive health.

Endocrine disrupting chemicals (EDCs) are chemicals which disrupt normal endocrine function by mimicking hormones or antagonising receptors, and have been implicated in abnormal reproductive development (Chevalier *et al.,* 2015). Studies where physiological concentrations of EDCs have been measured in study participants provide more conclusive results than correlative studies. Phthalates are one such example of EDC which are used in the manufacturing of numerous everyday products such as food packaging, shampoos and soaps, which confer flexible binding properties and have been highlighted as a concern for health. Phthalate concentration in urine has been associated with lower sperm concentration and sperm motility (Duty *et al.,* 2003; Jurewicz *et al.,* 2013), and a more thorough study

which investigated urinary phthalate levels on repeated occasions found higher levels of DNA damage and apoptosis in sperm with increasing exposure (Wang *et al.,* 2015). Bisphenol A (BPA) is another high volume EDC used in plastics manufacturing and has been found to be elevated in the urine of exposed factory workers and this correlated with decreased semen quality and increased DNA damage (Meeker *et al*., 2010). Pesticides are also considered to be a widespread source of EDCs. A recent study measured pesticide levels in the urine of men attending a fertility clinic to which sperm concentration and motility were negatively correlated (Melgarejo *et al.,* 2015), and in accordance with this the consumption of pesticide residues on fruit and vegetables has also been negatively correlated with sperm count and the number of morphologically normal forms (Chiu et al., 2015). Considering that EDCs have also been linked to birth defects and aberrant long term health and reproductive outcomes in children of exposed parents (Kristensen *et al.,* 1997; Wohlfahrt-Veje *et al.,* 2011; Anderson *et al.,* 2006), it is important to determine whether the use of ART increases the likelihood of the transmission of these developmental faults.

1.2.2.4 Diet

Three studies arising from the Rochester Young Men's Study found that certain dietary factors affect sperm quality. Firstly, dairy food intake was negatively correlated with sperm morphology (Afeiche *et al.,* 2013). Secondly, men with a "prudent" dietary pattern consisting of high intake of legumes, fish, chicken, fruit, wholegrains and vegetables had higher % progressively motile sperm (Gaskins *et al.,* 2012). Finally, consumption of sugar-sweetened beverages (SSB) was negatively correlated with % progressively motile sperm (Chiu *et al.,* 2014b). Critically, the reference population used for these studies was young healthy men (aged 18-22 years). This is important as it shows that dietary factors have an impact on men from the general population, in contrast to studies which recruit their study participants from fertility clinics.

1.2.2.5 Physical activity and sedentarism

The number of hours of physical activity that men participated in per week was directly correlated with sperm concentration and sperm count, while sedentary behaviour measured as hours of TV watched per week was negatively correlated with these outcomes (Gaskins *et al.,* 2015). Using taxi-drivers as a proxy for sedentary behaviour due to the long number of hours spent driving showed that these men had a lower % morphologically normal sperm forms, although exposure to pollutants could have been a confounding factor, as a result of the effects described above. Conversely, there is evidence that high strenuous physical activity can be damaging for reproductive function such as in the case of professional cyclists (Gebreegziabher *et al.,* 2004), and as was shown in an intervention study in which men who carried out intense treadmill running had decreased male reproductive hormone levels and lower sperm quality compared to a moderate exercise group (Safarinejad *et al.,* 2009). However, considering the overall widespread health benefits of exercise, it is important to highlight that in general being physically active is more beneficial for semen quality and reproductive function than being physically inactive (Vaamonde *et al.,* 2012).

1.3 Sperm epigenetics

Gene expression through the integration of transcription factors and modulators result in the synthesis of the messenger (m) RNA transcript from the DNA template. mRNA is then translated to the peptide sequence forming a vast variety of proteins for cellular structure and function. This canonical regulative control of gene transcription has been challenged by the emerging field of epigenomics.

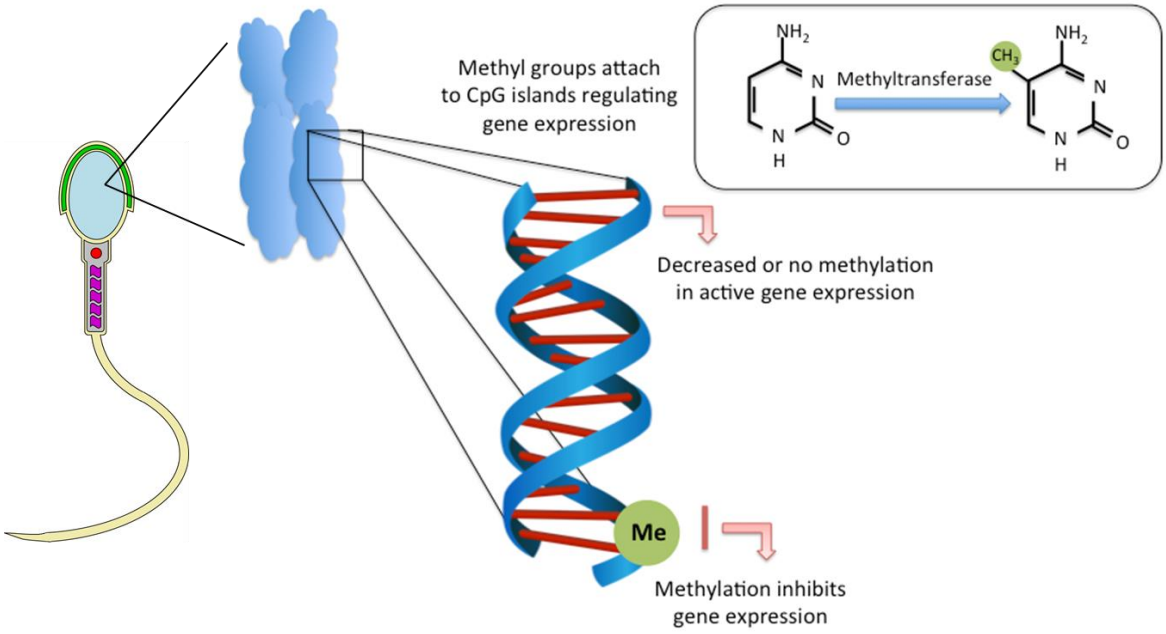


Figure 1.3 DNA methylation

The principle epigenetic modification of DNA in the mammalian genome is methylation of cytosine nucleotides. Methylation occurs mainly on the 5th carbon of the cytosine base, forming 5-methylcytosine (5-mC). DNA methylation is catalyzed by a family of enzymes called DNA methyltransferases and include DNMT1, DNMT3a and DNMT3b (box). Methylation mainly occurs around CpG clusters (CpG islands) at gene promoter regions and is associated with gene silencing.

Adapted from Nevin and Carroll, 2015

Epigenetics refers to the heritable changes in gene function by mechanisms other than changes in the underlying DNA sequence (Russo *et al.,* 1996). In contrast to traditional genetics in which mutation causes direct modification to the genetic sequence, epigenetics involves a complex set of regulatory machinery that function from the DNA to chromatin levels to influence gene expression, without affecting the fundamental nucleotide sequence itself (Jaenisch and Bird, 2003). Epigenetic control can therefore modify a phenotypic outcome without affecting the genotype. This process is the fundamental driving force of variation in cell type and function in cells during differentiation from zygote to multicellular organism. Historically, this was illustrated in Conrad Waddington's discovery of cell-fate determination by epigenetics (Waddington, 1957), which revealed the importance of

epigenetics in development. The molecular systems known to initiate and maintain the epigenome are DNA methylation and chromatin modifications, including histone methylation and acetylation (Murgatroyd and Spengler, 2011). There is a critical cross-talk between this epigenetic machinery which ensures correct gene expression and silencing in response to environmental cues (Mutskov *et al*., 2002; Curradi *et al.,* 2002).

1.3.1 DNA Methylation

DNA methylation is the most well-studied epigenetic mechanism in mammals (Jones and Takai, 2001). It is a major regulator of gene expression and cell differentiation (Li, Bestor and Jaenisch, 1992), and therefore the capacity for DNA methylation marks to be stably maintained (i.e. somatically inherited) through cell division is the fundamental principle behind differentiation and the processes in which cells are able to interact with the environment. DNA methylation occurs on the 5' position of cytosine bases on eukaryotic DNA. Cytosines targeted for methylation usually occur in CpG dinucleotides, where a cytosine precedes a guanine nucleotide, although cytosine methylation can also occur at CpA, CpT and CpC sites (Maunakea *et al.,* 2010). CpG sites are mostly located close together in clusters known as CpG islands (CGIs), and are commonly found in the 5' promoter region of genes or in gene bodies (Lander, Linton and Birren *et al.,* 2001; Maunakea *et al.,* 2010).

The process of DNA methylation is driven by the action of the DNA methyltransferases (DNMTs) DNMT1, DNMT3a and DNMT3b which convert cytosine to 5-methylcytosine (5mC) using the universal methyl donor S-adenosylmethionine (SAM) (Okano *et al*., 1999) (fig. 1.3 box). DNMT1 is the "maintenance" methyltransferase as its primary role is to copy methylation patterns onto newly replicated DNA, while DNMT3a and DNMT3b are responsible for *de novo* methylation of DNA (Kaneda *et al.,* 2004a). These enzymes are essential for viability as gene knockouts experiments in mice have shown embryonic and postnatal lethality (Li, Bestor and Jaenisch, 1992; Okano *et al.,* 1999).

CpG methylation at promoters or regulatory regions is typically associated with gene repression while lack of methylation promotes transcriptional activity (Siegfried et al., 1999; Polansky et al., 2008). Transcriptional repression is caused either through the direct blocking of transcription factor binding to DNA, or through the attraction of methyl-CpG-binding protein which recognise methylated sites and subsequently recruit co-repressors (Watt and Molloy, 1988; Nan *et al*., 1998; Boyes and Bird, 1991) (fig. 1.3). DNA methylation has a number of crucial roles in embryonic development and differentiation (Okano *et al.,* 1999), X-chromosome inactivation (Kaneda *et al.,* 2004b), suppression of transposable element activity (Nagamori *et al.,* 2015) and genomic imprinting (Kaneda *et al.,* 2004b).

1.3.2 Epigenetic reprogramming and imprinted genes

Epigenetic processes in the gametes of mammals are different to those in somatic cells. During development, DNA undergoes two rounds of epigenetic reprogramming (fig. 1.4). The first is shortly after fertilisation, where the paternal DNA is stripped of the parental methylation signature. This global demethylation occurs in sperm and oocyte DNA (Guo *et al.,* 2014; Oswald *et al*., 2000; Santos and Dean, 2004) and permits the totipotent zygote to initiate new gene transcription (Hacket and Surani, 2013).

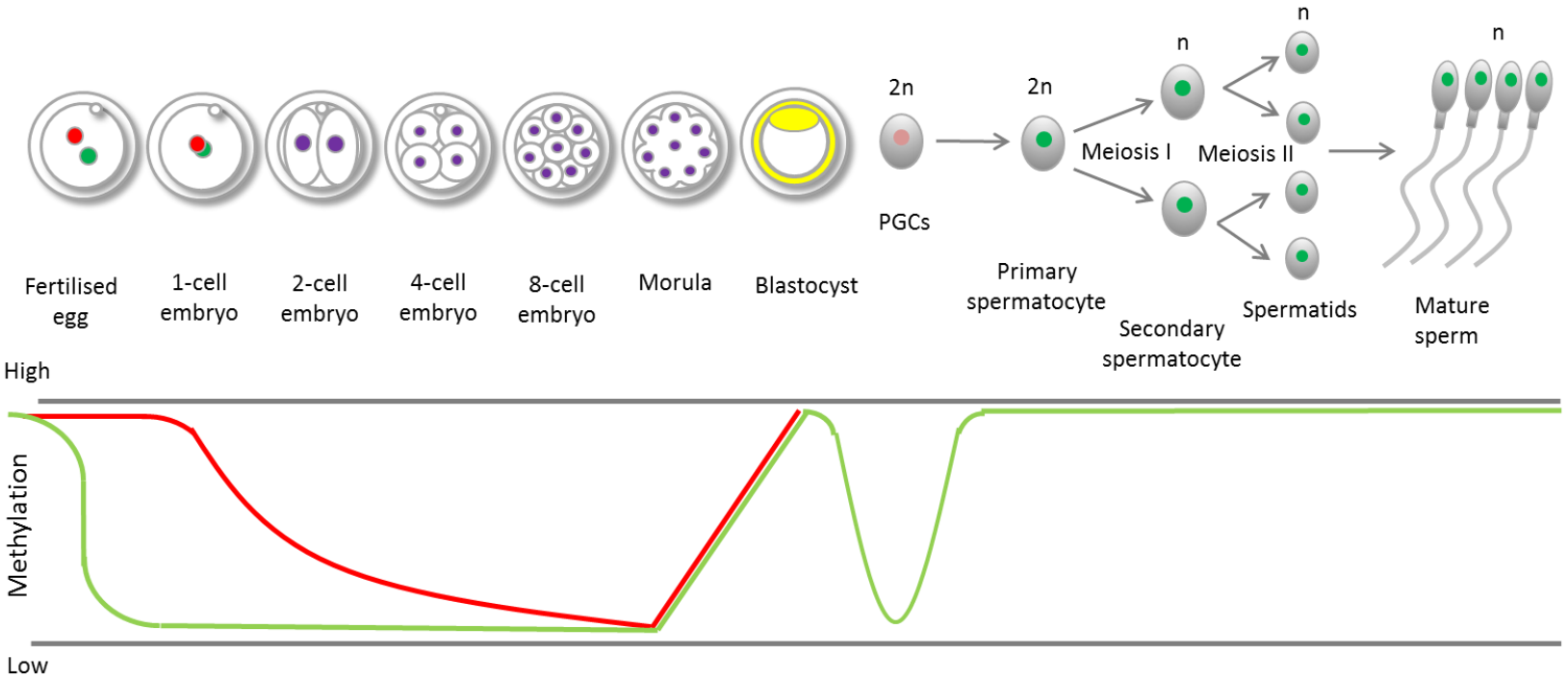


Figure 1.4 Epigenetic reprogramming

The paternal DNA (green) is rapidly demethylated following fertilisation while maternal DNA methylation (red) is maintained until the 2-cell stage when it is passively removed. Primordial germ cells undergo rapid global demethylation after their migration into the gonadal ridge to remove parental imprints and allow establishment of gender-specific imprints.

Adapted from Nevin and Carroll, 2015

These processes allow the developing zygote to initiate de novo methylation in cells of the inner cell mass (Santos *et al*., 2002) to regulate the expression of genes important in the differentiation pathways leading to the specialisation of cell types.

Despite this global demethylation, some genes are excluded and retain the methylation marks of the parental genome. These genes are known as imprinted genes, and are critical for embryonic development during the early rounds of transcription in the embryo. Imprinted genes show mono-allelic parent-of-origin expression: they are only expressed from one parental allele (Court *et al*., 2014). The importance of this imprinting was illustrated in early pro-nuclei transfer experiments carried out by McGrath and Solter (1984). They found that when two haploid male pronuclei or two haploid female pronuclei were combined, although each genome was contributing half of the genetic information, there were sex-specific epigenetic marks corresponding to paternal or maternal DNA that were critical for embryonic development. Imprinted genes escape demethylation so that correct gene expression can be initiated in the developing embryo, hence why their mutation results in severe developmental imprinting disorders (Reik and Walter, 2001; Soejima and Higashimoto, 2013).

These imprints continue to persist in somatic cells after fertilisation, however developing germ cells must undergo a second round of reprogramming to allow the establishment of new imprinting regions (Reik and Walter, 2001; Kaneda *et al*., 2004b). PGCs have already acquired regional DNA methylation on their migration to the gonadal ridge (precursor to the gonads), similar to somatic cells (Maatouk *et al*., 2006), and demethylation therefore allows establishment of mature germ cell gender-specific genetic imprints (Hajkova *et al*., 2002). The establishment of male imprints are complete in prospermatogonia by the newborn stage and global methylation of repeat sequences are highly methylated by the fetal prospermatogonia stage (Sasaki and Matsui, 2008).

Imprinted genes are controlled by differentially methylated regions (DMRs) at CpG sites or CpG islands within or close to the gene itself (Neumann *et al.,* 1995). If a gene is paternally imprinted, its DMR is heavily methylated in sperm while unmethylated in oocytes, whilst a maternally imprinted gene is unmethylated in sperm but methylated in oocytes. Maternally and paternally imprinted genes usually exist in clusters where their expression is controlled by DNA methylation at cis-regulatory sites known as imprinting control

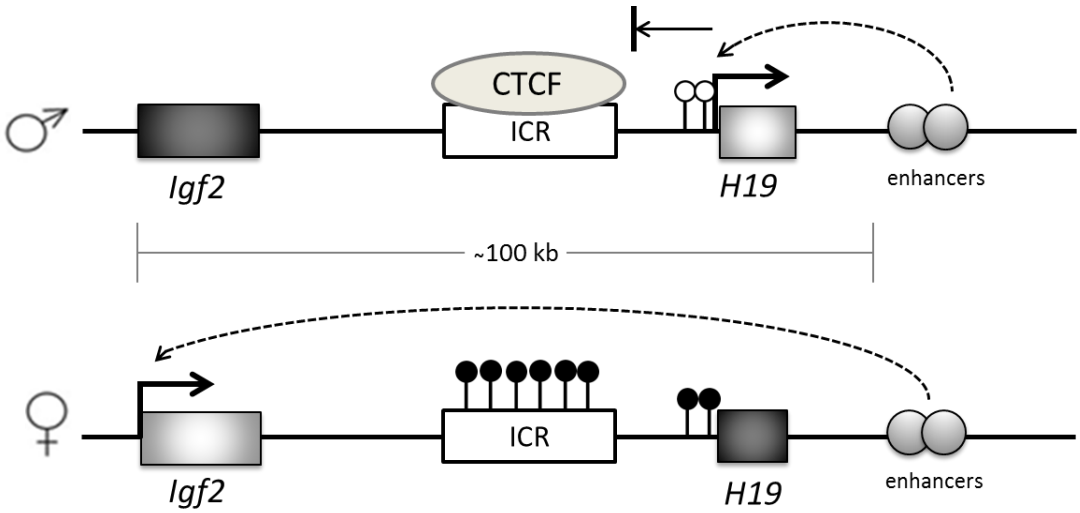


Figure 1.5 Methylation at the *IGF2-H19* imprinting control region (ICR)

Imprinted genes exhibit parent of origin gene expression. This is achieved through methylation-dependent gene silencing at imprinting control regions (ICR). On the maternal allele at the *IGF2-H19* locus the ICR is not methylated, allowing CTCF binding which silences *IGF2* gene transcription through downstream enhancer activity. Maternally unmethylated *H19* promoter (white lollipops) allows *H19* transcription. On the paternal allele, CpG sites are methylated (black lollipops) on the ICR and in the *H19* promoter region. This prevents CTCF binding, which allows enhancers to activate *IGF2* while silencing *H19.*

Adapted from Nevin and Carroll 2015

regions (ICR). The methylation state of the ICR determines the methylation status of the genes in the cluster either over short- or long-range (Lin *et al*., 2003).

One of the best characterised ICRs is at chromosome 11q15 which contains the insulin-like growth factor 2 (*IGF2*) and the downstream non-coding RNA gene, *H19*, which are expressed from the paternal and maternal chromosomes, respectively. Their expression is controlled by an enhancer downstream of *H19*, which is in turn controlled by methylation at an ICR located between the two genes (Hark *et al*., 2000) (fig. 1.5). The ICR contains a number of binding sites for the transcriptional repressor, zinc finger CCCTC-binding factor (CTCF), which in the absence of methylation can bind to the ICR and activate the downstream enhancer. This in turn activates *H19* transcription while *IGF2* is silenced. When the ICR is methylated, as it is in sperm, CTCF cannot bind and the downstream enhancers are free to activate *IGF2* expression while *H19* becomes silenced (Kerjean *et al*., 2000). Beckwith-Weidemann syndrome is an imprinting disorder which arises from the loss of imprinting at the *IGF2-H19* locus. When uniparental disomy occurs, both alleles are inherited from one parent. In the case of Beckwith-Weidemann syndrome, both alleles come from the father and none from the mother (Henry *et al*., 1991). This results in hyperactivation of *IGF2* as the ICR is methylated on both alleles. As *IGF2* is a growth promoter, the disease is characterised by serious foetal and neonatal overgrowth as well as a predisposition to tumour growth (Morison and Reeve, 1998).

Another example of an important ICR is located at chromosome 14q32 at the intergenic (IG) region between the paternally expressed (i.e. maternally imprinted) delta-like 1 homolog (*DLK1*) gene and the maternally expressed gene 3 (*MEG3*) (i.e. paternally imprinted) (Kagami *et al.,* 2010). This region bares similarities with the *IGF2-H19* locus as it contains a CTCF binding region (Wylie *et al.,* 2000). The IG-DMR is methylated on the paternal chromosome and unmethylated on the maternal chromosome, and inheritance of the paternal allele as a result of maternal deletion results in complete silencing of *MEG3* and overexpression of *DLK1* resulting in a lethal phenotype (Lin *et al.,* 2003). Numerous other genes are controlled through this ICR and loss of imprinting therefore causes severe developmental defects in humans (Kagami *et al.,* 2010). Furthermore, aberrant imprinting of

this region is considered to be a possible marker for predisposition to type 1 diabetes (Wallace *et al.,* 2010).

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are examples of imprinting disorders that occur due to opposing errors on the same locus on chromosome 15q11-q13 (Buiting *et al.,* 2003). PWS occurs as a result of a deletion of part of the paternal chromosome or as a result of maternal uniparental disomy, while AS occurs as a result of maternal chromosome deletion. This region contains a number of genes that are maternally imprinted and exclusively paternally expressed, including the small nuclear ribonucloprotein polypeptide N (*SNRPN*) gene (Reed and Leff, 1994). Therefore, deletions of the paternal allele results in no expression of this gene leading to the PWS phenotype. In a small number of cases, PWS and AS can arise due to methylation errors, or epimutations, at the imprinted genes within this region, instead of causing a change to the DNA sequence. These epimutations are likely to arise due to improper reprogramming during development (Buiting *et al.,* 2003).

There are over 100 known imprinted genes in humans (Kelsey and Bartolomei, 2012), most of which are involved in foetal, placental and brain development, post-natal growth, behaviour and metabolism (Tycko and Morison, 2002). Imprinting ensures that a critical balance of gene dosage is maintained when parental chromosomes act together and its disruption can lead to abnormalities in male fertility as well as the severe developmental defects discussed.

1.3.3 DNA methylation in sperm function and male infertility

The identification of aberrant methylation in the sperm of infertile men has led to speculation that deregulated epigenetic control may be one of the causes of infertility. The differentiation of spermatids into mature sperm occurs simultaneously with remethylation of the genome following the second wave of reprogramming where new imprints are laid down (Kishigami *et al*., 2006; Oakes *et al*., 2007) (fig. 1.4). Disruption of methylation at this

point may therefore be a source of abnormal germ cell development, leading to MFI.

Most studies investigating the association between aberrant DNA methylation and infertility have focused on candidate imprinted genes (Poplinski *et al.,* 2010; Kobayashi *et al*., 2007; Minor *et al*., 2011; Ankolkar *et al.,* 2013; Boissonnas *et al.,* 2010). This is because imprinted genes in germ cells have unique reprogramming events which means they show specific methylation signatures, and due to the importance of paternally imprinted genes in post-zygotic gene expression (Carrell and Hammoud, 2010).

Initial epigenetic studies comparing imprinted gene methylation in sperm DNA of oligozoospermic men, in comparison with normozoospermic men, have tended to show a decrease in methylation of paternally imprinted genes such as *H19* and an increase in maternally imprinted genes such as mesodern-specific transcript (*MEST*) (Marques *et al.,* 2007). Specifically, loss of methylation at the 6th CTCF binding site upstream of *H19* has been associated with severe sperm defects in morphology, motility and concentration (Marques *et al*., 2007; *Boissonnas et al.,* 2010). Others have corroborated this methylation pattern seen in the *H19* gene in further studies with oligozoospermic men (Poplinski *et al*., 2010; Kobayashi *et al.,* 2007; Montjean *et al*., 2013). Interestingly, Minor *et al.* (2011) found that sperm retrieved from the testes of men with obstructive azoospermia also demonstrated hypomethylation of *H19*, suggesting that aberrant methylation may be a product of the testicular environment and not singularly due to spermatogenesis failure. *MEG3,* also known as *GTL2,* is highly methylated in sperm (Geuns *et al.,* 2007) and has also been shown to be hypomethylated in oligozoospermic men compared to normal men, similar to that of *H19* (Kobayashi *et al.,* 2007; El Hajj *et al.,* 2011).

Maternally imprinted genes which normally show low levels of methylation in sperm have exhibited increases in methylation in men with poor sperm quality. *MEST* hypermethylation has repeatedly been linked to low sperm count, low progressive sperm motility and poor sperm morphology (Poplinski *et al*., 2010; Kläver *et al.,* 2013; Houshdaran *et al*., 2007). *MEST* is a

candidate gene for the developmental disorder Silver-Russel Syndrome (SRS) and interestingly, in leukocyte DNA from a patient with SRS, Kagami (2007) found hypermethylation at CpG sites in the *MEST* gene occurred at a number of the same sites in the paternal DNA, suggesting improper reprogramming in the offspring and a heritability of some paternal methylation marks. Similarly, the *SNRPN* gene was found to be hypermethylated in oligozoospermic patients as well as patients with abnormal protamine ratios (Hammoud *et al.,* 2010), However *SNPRN* hypomethylation has been associated with infertility elsewhere (El Hajj *et al.,* 2011). Increased methylation of pleiomorphic adenoma gene like 1 (*PLAGL1*), also maternally imprinted, has been associated with poor sperm quality (Houshdaran *et al.,* 2007), and proper methylation of this gene is critical for prenatal and post-natal growth in healthy infants (Azzi *et al.,* 2014).

Studies mostly focus on a particular set of genes and gene-regulatory regions to determine how lifestyle and environment affect methylation, as these are the sites which are most likely to affect their associated gene's expression. However, investigating global DNA methylation has been important for determining genome-wide changes to DNA including at intergenic regions and at repetitive elements (Maunakea *et al.,* 2010). The short-interspersed element, *ALU*, and the long interspersed element 1 (*LINE-1*) are examples of repeat elements which are used as markers for global methylation (Chalitagorn *et al.,* 2004; Yang *et al.,* 2004). They comprise 10% and 17% of the human genome, respectively and therefore largely represent epigenetic changes at multiple loci (International Human Genome Sequencing Consortium, 2004). These elements have retrotransposon activity which means they can replicate and move about the genome if uncontrolled, leading to disrupted function of other genes. Therefore they are heavily methylated to prevent this activity (Yoder *et al.,* 1997). *LINE-1* and *ALU* methylation has been used as a marker for global methylation in sperm, and *ALU* hypomethylation has been linked to poorer sperm quality and negative ART outcomes (El Hajj *et al.,* 2011); this may be therefore be more informative than imprinted gene methylation. Another method for the

determination of global methylation is the use of enzyme-linked immunosorbent assays (ELISA) to measure 5-methyl-cytosine (5MedCyd) concentration. As with the repeat elements, 5MedCyd concentration has also been found to be positively correlated with sperm parameters (Montjean *et al.,* 2014). However, not all studies have reported significant differences in global methylation between fertile and infertile men (Kobayashi *et al*., 2007; Ankolkar *et al*., 2013; Boissonnas *et al.,* 2010).

Aberrant DNA methylation at imprinted genes has been widely associated with sperm abnormalities and infertility, as discussed. However, methylation at non-imprinted sites may also be important for expression of the paternal genome in the developing embryo. High throughput array technology has allowed the exploration of methylation across the whole genome, and has resulted in the finding that many non-imprinted genes are also linked to poor semen parameters (Houshdaran *et al*., 2007; Schütte *et al.,* 2013).

Methylenetetrahydrofolate reductase (*MTHFR*) is a major regulatory gene in folate metabolism, remethylation and DNA synthesis. It is vital for providing sufficient methyl groups for DNA synthesis and methylation and is thought to have an important role in spermatogenesis due to its association with low sperm count and non-obstructive azoospermia (Khazamipour *et al*., 2009; Singh *et al.,* 2005). *MTHFR* promoter hypermethylation has been strongly associated with idiopathic male infertility (Wu *et al*., 2010), and interestingly, this hypermethylation has been shown to occur alongside hypomethylation of *H19* (Rotondo *et al.,* 2013). Given the role of MTHFR in normal methylation, its deregulation could have knock-on effects for other genes. Not only has *MTHFR* hypermethylation been associated with male infertility, but also with spontaneous abortion rates (Rotondo *et al.,* 2012), highlighting the potential consequences of aberrant methylation.

Spermatogenesis genes are also susceptible to DNA modifications and can be directly linked to male infertility. Deleted in azoospermia-like (*DAZL*) is a key spermatogenesis gene important for primordial germ cell formation, while it is also expressed in mature sperm (Kee *et al*., 2009; Lin *et al.,* 2002). Single nucleotide polymorphisms (SNP) as well as aberrant methylation in

the promoter of *DAZL* are associated with abnormal semen parameters (Teng *et al*., 2012; Navarro-Costa *et al*., 2010), indicating that this is an important gene for male fertility. *DAZL* has been found to be abundantly expressed in the pre-implantation embryo from the 2-cell stage, however this is thought to come through maternal genome control, and so the contribution of paternal *DAZL* methylation is yet to be determined (Cauffman *et al*., 2005).

As 50% of male infertility cases have an unknown cause, this suggests that it is in fact a multifactorial disease. As DNA methylation is an important regulator of biological processes, including spermatogenesis, and given the examples of aberrant methylation in infertile men, it is likely that this epigenetic mechanism plays a part.

1.3.4 Lifestyle and environmental effects on sperm DNA methylation

The crucial feature of the epigenome is that it is receptive to the environment; this is the fundamental principle behind the cellular response to external stimuli. However, it also means that damaging environmental and lifestyle factors can negatively influence DNA methylation and consequently cause aberrant gene expression, leading to cellular dysfunction and disease (Murgatroyd *et al*., 2010; Jones and Baylin, 2002). Evidence that lifestyle and environmental factors can alter sperm DNA methylation suggests that these factors may contribute to MFI.

1.3.4.1 Age

The effects of paternal age on semen quality and reproductive outcomes have been explored for years with variable findings. A 2010 review confirmed that there are significant reductions in semen parameters with increasing age, and that paternal age is also associated with reduced fertility, pregnancy complications, and adverse childhood health outcomes (Sartorius and Neischlag, 2010). Global DNA methylation, as well as gene-specific methylation, changes across different cell types and tissues with advancing age (Hovarth, 2013). This is also the case for sperm, as was revealed by one

study which looked at sperm DNA methylation in the same individuals 10 years apart: the authors found subsets of genes exhibiting age-related hypo- and hypermethylation (Jenkins *et al.,* 2014). As such, it is possible that deregulated gene expression through changes in the epigenome could contribute to the age-related declines in fertility. Interestingly, Adkins *et al*. (2011) found correlations between methylation at a number of CpG sites in newborns and paternal age, indicating that age-induced paternal epigenetic modifications may be maintained in the embryo.

1.3.4.2 Obesity

There is considerable evidence to show that obese individuals exhibit altered DNA methylation at numerous gene promoters and CpG islands in blood samples (Ding *et al.,* 2015). Obesity has been found to be associated with aberrant sperm methylation in some, but not all, studies (Donkin *et al.,* 2015; Hesson *et al.,* 2015). Although the evidence for the effects on sperm DNA methylation is inconclusive, imprinted genes have been shown to be hypomethylated in the children of obese fathers compared with normal weight fathers (Soubry *et al*., 2013). This suggests that obesity does induce some epigenetic changes. Critically, this disruption of DNA methylation profiles in newborns can have implications for future health, as illustrated by Godfrey *et al.* (2011) who found that altered methylation in adiposity-related genes at birth was related to adolescent obesity. This research suggests that there may be a preconceptual influence of paternal health on long-term gene regulation in offspring. Interestingly, the changes in DNA methylation as a result of obesity could be reversible as one study found positive changes in sperm DNA methylation in men on an exercise intervention programme (Denham *et al.,* 2015).

There is considerable interest in whether paternal germline epimutations are heritable as this suggests a much more important role for paternal lifestyle and environmental exposures than previously thought. One study, which investigated DNA methylation in the offspring of obese fathers, found that a DMR involved in control of the *IGF2* gene was found to be hypomethylated in

umbilical cord blood leukocyte DNA of offspring from obese fathers (Soubry *et al*., 2013). Longitudinal health studies have also suggested a role for paternal epigenetic inheritance. The pre-pubescent slow growth period (SGP) in males coincides with the emergence of the first viable pools of spermatocytes where the cells may be particularly vulnerable to epigenetic change. A study of a large cohort of Swedish males found increased mortality rates of individuals whose grandfathers experienced over-nourishment during the SGP (Pembrey *et al.,* 2006). Concurrently, cardiovascular mortality rates were found to be lower in men whose grandfathers experienced a restricted food supply during the SGP, yet when food supply was in excess, diabetes mortality in grandchildren was high (Kaati, Bygren and Edvinson, 2002). These studies highlight a possibility for epigenetic inheritance through multiple generations, and DNA methylation could be a mechanism for this.

Mouse models have been an insightful platform for the study of the transgenerational epigenetic effects of nutrition on offspring health and disease. Overfeeding and starvation of male mice prior to mating can result in metabolic deregulation in their offspring (Jimenez-Chillaron *et al*., 2009; Binder *et al*., 2012). A recent seminal study that sought to determine whether these characteristics are transmitted through the epigenome found that induced paternal prediabetes led to altered methylation in the sperm DNA. This caused altered gene expression in glucose uptake and metabolism genes and insulin signalling genes in the offspring, leading to glucose intolerance and insulin resistance (Wei *et al.*, 2014). Furthermore, Fullston *et al.* (2013) found that the effects of paternal obesity could be detected as far as the F2 mouse generation and affected sperm mRNA profiles as well as DNA methylation. Therefore, paternal environmentally-acquired epigenetic changes, such as through obesity, could have serious implications for offspring health.

1.3.4.3 Smoking

Cigarette smoking is considered to be one of the major environmental modifiers of CpG methylation (Breitling *et al*., 2013). Smoking has been shown to reduce DNA methylation at multiple loci, and critically, these modifications can be partially reversed on smoking cessation (Tsaprouni *et al*., 2014). It is well known that prenatal maternal smoking has serious implications for developmental gene expression, and consequently offspring health (Markunas *et al*., 2014; Richmond *et al*., 2015), however, the role of paternal smoking in DNA methylation in offspring is less understood. Xu *et al*. (2013) found significant differences in gene-specific methylation in sperm DNA of mice exposed to cigarette smoke, however, in humans paternal smoking was not found to be linked to altered *H19* or *IGF2* DMR methylation in umbilical cord DNA of newborn children (Bouwland-Both et al., 2015).

1.3.4.4 Environmental toxicants

One of the causes of MFI through environmental exposures is likely to be through the hormonal deregulation caused as a result of exposure to EDCs. Aberrant DNA methylation of sperm DNA may occur as a result of this disruption or through a separate mechanism. *LINE-1* hypomethylation has been detected in the sperm of men exposed to BPA compared with non-exposed men (Miao *et al*., 2014). Mouse studies, again, have shed light on the transmission of these epigenetic modifications through the male germline: *Igf2* DMR methylation was perturbed in the F2 males of pregnant mice exposed to BPA resulting in metabolic dysfunction (Susiarjo *et al*., 2015). Animals exposed to vinclozolin and dichlorodiphenyltrichloroethane **(**DDT) have also demonstrated generational epigenetic modifications and associated diseases (Guerrero-Bosagna *et al.,* 2012; Skinner *et al*., 2013). However, the extent of this epigenetic memory in humans in response to environmental chemicals is yet to be elucidated.

1.3.5 Imprinting defects and ART

With the use of ART treatment increasing (European Society of Human Reproduction and Embryology, 2014), there is concern over the use of subquality sperm during these procedures, which may harbour DNA damage or aberrant DNA methylation marks. There is also concern over the effects of ART methods themselves on gamete DNA integrity, as cells are exposed to various culture conditions *in vitro,* alongside female hormonal stimulation and cryopreservation of sperm and oocytes. This concern has arisen due to the reported increase in the frequency of imprinting disorders, such as BWS, in children born through ART, which are normally extremely rare in the general population (Lazaraviciute *et al*., 2014; Olivennes *et al*., 2001). Therefore, ART may present some risks to gamete DNA integrity.

Cryopreservation of sperm and oocytes is a routine process in ART procedures and has become more popular as couples delay parenting until later in life. Studies in mice have linked oocyte vitrification to reduced methylation at imprinted genes in blastocysts, and this was attributed to reduced DNMT expression (Cheng *et al*., 2014). This was partially supported in a human oocyte cryopreservation study (Petrussa *et al.,* 2014). Human sperm cryopreservation, however, has been reported to be completely safe, conferring no changes to methylation at imprinted genes, spermatogenesis-related genes or *MTHFR* in the sperm DNA (Kläver *et al*., 2012). Ovarian stimulation has also been linked to altered imprinted gene methylation of embryos in some cases, but not in others (Sato *et al.,* 2006; Market-Velker *et al.,* 2010; Denomme *et al.,* 2011).

There are a number of debates ongoing with regards to the impact of aberrant sperm methylation on ART outcomes. The first concerns whether sperm imprinting defects are associated with poorer ART outcomes (Ankolkar *et al.,* 2013; Camprubi *et al.,* 2012). The second is whether aberrant methylation marks are inherited and may affect embryonic development and offspring health. Kobayashi *et al.* (2009) found that some imprinted gene methylation errors in conceptuses conceived through ART could be directly associated with the paternal genome. Conversely, although

Lazaravicuite *et al*. (2014) found higher rates of imprinting disorders in ART compared with non-ART children, they could not identify any significant methylation differences at specific imprinted genes between these groups. The extent of the contribution of DNA methylation to male infertility and germline inheritance is still unclear. However, as the epigenome evidently acts as the connection between the environment and gene regulation, lifestyle and environmental interventions may play some part in improving fertility and preventing offspring disease.

1.4 Advanced glycation end products and male reproductive function

Advanced glycation end products (AGEs) are a heterogeneous group of compounds that form as a result of the reaction between sugars and biomolecules, and are the cause of a number of diabetes- and age-related diseases where AGE levels are elevated. These diseases include cardiovascular disease, diabetic nephropathy, nerve damage, retinopathy and atherosclerosis (Hanssen *et al.,* 2015; Forbes and Cooper, 2012; Duran-Jiminez *et al.,* 2009; Ahmed *et al.,* 1997; Stitt *et al.,* 1997). Ultimately, AGEs are thought to be an important contribution to the multisystem functional decline in health that occurs with ageing. Obesity, diabetes and ageing have been linked to declines in semen quality and fertility (Eisenberg *et al.,* 2014; Sartorius and Neischlag, 2010). Despite the prevalence of AGEs in these conditions, there is very little research on their effects on reproductive function. So far, AGEs have been located in the male reproductive tract, on sperm cells and in soluble form in the seminal plasma (Mallidis *et al.,* 2009; Karimi *et al.,* 2011). This suggests that they may form modifications on functionally important sperm proteins or DNA adducts, or additionally as AGE formation is implicated in ROS production, this could be a source of oxidative DNA damage to sperm (Mallidis *et al*., 2009).

1.4.1 Structure and function of AGE

AGEs are formed *in vivo* from the spontaneous non-enzymatic reaction of reducing sugars with proteins, lipids and nucleic acids, through the Maillard reaction, lipid peroxidation and the polyol pathway (Ott *et al*., 2014). AGE adducts accumulate in the body over time as a normal part of the ageing process; however their formation is accelerated under hyperglycaemic conditions, insulin resistance, dyslipidemia and due to oxidative stress in diabetes (Ulrich and Cerami, 2001; Thomas *et al*., 2005; Unoki and Yamagishi, 2008). AGE compounds are also formed exogenously from the cooking of foods at high temperatures and in tobacco smoke (Uribarri *et al.,* 2010; Cerami *et al.,* 1997), meaning that lifestyle and environment can introduce high levels of AGE to the body. Endogenous and exogenous AGE interact with the cell membrane receptor for AGE (RAGE), initiating downstream inflammatory signalling (Cerami *et al*., 1997).

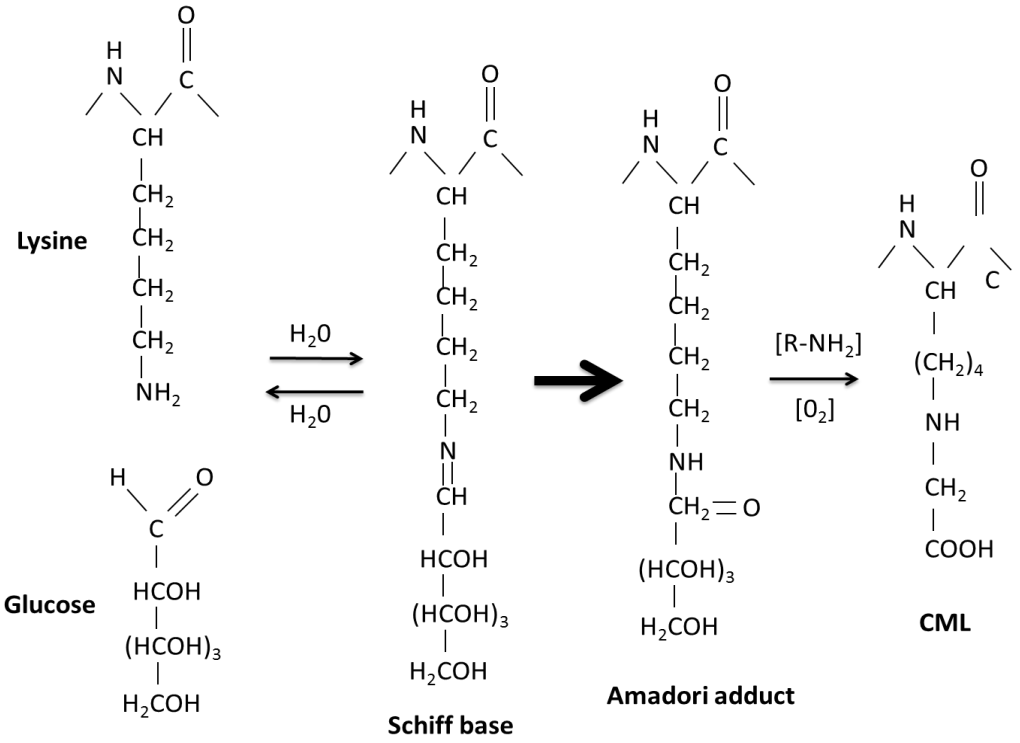


Figure 1.6 Glycation of lysine by glucose

The carbonyl group of glucose reacts with the amine group of a lysine residue, forming a reversible Schiff's base adduct. A number of rearrangements occur to form an Amadori product. This then undergoes further glycation and oxidation reactions to form Nε-(carboxymethyl)lysine (CML).

Adapted from Bohlender, 2005

1.4.2 Formation and action of AGEs

Glycation incorporates a chain of reactions known as the Maillard reaction which begins with the non-enzymatic reaction of a carbonyl group from a reducing sugar, with free amino groups in protein, lipid or nucleic acids (Goldin *et al.,* 2006). AGEs form as a normal part of metabolism as glucose reacts with biomolecules, and their accumulation in the body is a normal part of aging. However, under conditions of hyperglycaemia, hyperlipidaemia and

under oxidative conditions, AGE formation is accelerated and becomes pathogenic (Singh *et al*., 2001). AGE formation occurs on extracellular and, more rapidly, on intracellular proteins (Giardino, Edelstein and Brownlee, 1994; Thornalley *et al.,* 2003).

The first stage of the Maillard reaction is the condensation reaction between the carbonyl group of a reducing sugar, such as glucose, with the free amino group of a protein, initially forming a Schiff's base which is completely reversible. The main residues in proteins targeted for this are arginine and lysine. Schiff base levels are maintained at equilibrium with the surrounding glucose concentration (Brownlee *et al.,* 1984). Covalent modifications then more slowly turn the Schiff's base into a stable Amadori product (fig. 1.6). The most familiar Amadori products are fructosamine and glycated haemoglobin (HbA_{1c}) which are used widely as markers for serum glucose levels. These products are known as early-glycation adducts and are reversible although the rate of formation is faster than the rate of the reverse reaction (Brownlee *et al.,* 1984). To form end-stage glycation adducts, or AGEs, Amadori products undergo a number of further reactions including condensation, fragmentation and rearrangements that reorder their functional groups and these may occur under oxidative and non-oxidative conditions (Booth *et al.,* 1997). This results in the formation of stable irreversible adduct formation on the target molecule. AGEs cause damage by generating ROS, fluorescing, forming cross-links and binding to cell surface receptors (Thornalley, 1998). One of the major AGE-protein epitopes is N-ε-carboxymethyl lysine (CML), formed on lysine residues as a result of sequential glycation and oxidative reactions of Amadori products (Booth *et al.,* 1997) (fig. 1.6). The presence of CML in numerous tissues, including the male reproductive tract, suggests it could be involved in several AGE-associated pathologies. In addition, CML is formed earlier on in the Maillard reaction than other non-CML AGEs, making it a useful AGE compound to target for short *in vitro* studies. As the most prevalent biological sugar, glucose levels are the major contributor to the Maillard reaction, however other reducing sugars such as fructose, ribose and glucose-6-phosphate can react in a similar way to result in numerous chemically distinct AGEs.

AGEs can be broadly grouped into three categories: firstly, fluorescent cross-linking AGE such as pentosidine; secondly, non-fluorescent cross-linking AGEs such as imidazolium dilysine cross-links; and finally, non-crosslinking AGEs such as pyrraline and CML.

AGEs can also be generated from through the polyol pathway, also known as the sorbitol pathway, which shows prevalent activity in diabetes (Oates *et al.,* 2002). This is a two-step enzymatic pathway in which glucose is firstly converted to sorbitol by aldose reductase and then oxidised to fructose by sorbitol dehydrogenase (fig. 1.7). This reaction is also elevated as a result of hyperglycaemia and the enzymes required for the reaction are elevated in diabetic tissues (Lorenzi, 2007). The pathway eventually generates AGEs as

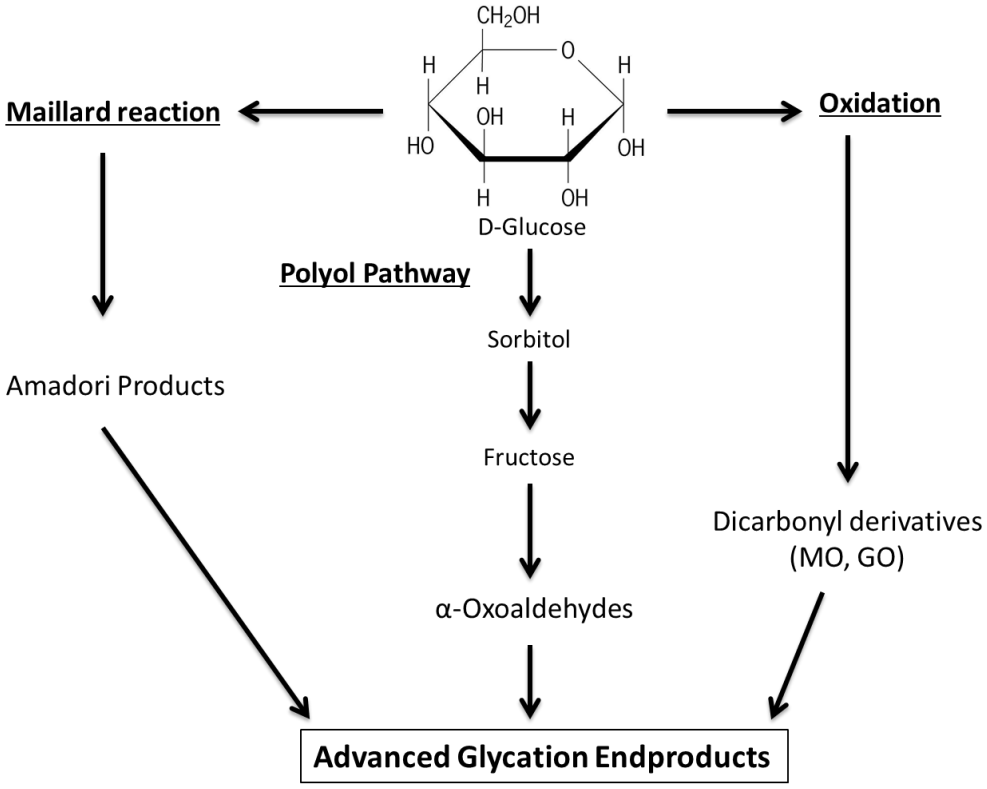


Figure 1.7 Formation of advanced glycation end products

AGEs form from the non-enzymatic glycation of proteins by glucose through the Maillard reaction, or through the enzymatic polyol pathway, or through autoxidation of glycose. Reactive AGE intermediates are formed from all pathways.

fructose is phosphorylated and broken down into 3-deoxyglucosone (3-DG), an AGE precursor (Szwergold *et al.,* 1990). 3-DG is a potent AGE-intermediate that rapidly forms AGE-adducts (Hamanda *et al.,* 1996). Furthermore, AGEs can also be generated from the autoxidation of monosaccharides including glucose.

As well as forming Amadori products, a Schiff's base can also fragment to generate reactive AGE intermediates, namely α-oxoaldehydes or α-dicarbonyls, which are about 20,000 times more reactive than glucose in glycation reactions, and these compounds themselves react with proteins, lipids and nucleic acids to form AGEs (Thornalley, 1996). These

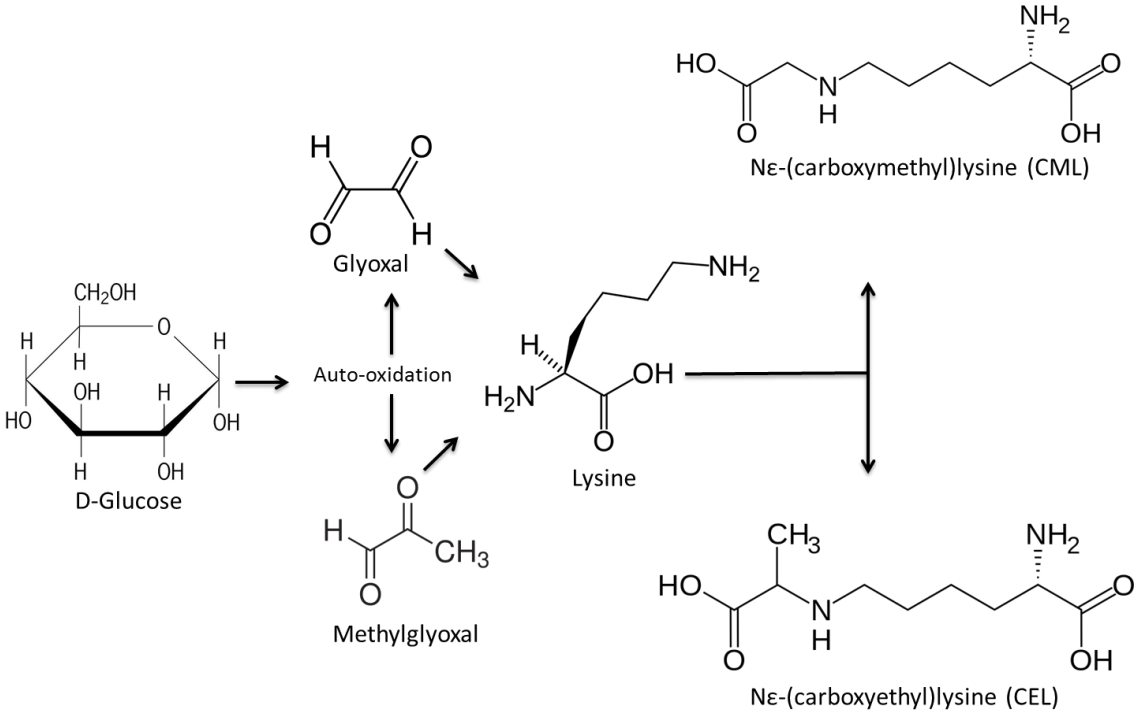


Figure 1.8 Formation of AGEs by methylglyoxal and glyoxal

The dicarbonyl compounds methylglyoxal (MG) and glyoxal (GO) are reactive intermediates produced by cell metabolism, glucose autoxidation, and lipid peroxidation.

These highly reactive compounds may react with amino and sulfhydryl groups in proteins to form irreversible advanced glycation end products (AGEs). Nε-(carboxymethyl)lysine (CML) and Nε-(carboxyethyl)lysine (CEL) are the major AGE adducts formed from GO and MGO, respectively.

intermediates include methylglyoxal (MG), glyoxal (GO) and 3-DG. MG and GO are also formed from the degradation of proteins glycated by glycose and from the autoxidation of monosaccharides (Thornalley *et al.,* 1999) (fig. 1.8). This widespread formation of AGE intermediates means they are present in almost all cases where AGEs are present. The rapid glycating activity of these intermediates makes them a useful tool for *in vitro* glycation experiments. When these intermediate react with proteins, they can form a range of products. MG predominantly reacts with lysine residues to form the minor AGE Nε-(1-carboxyethyl)lysine (CEL) (fig. 1.8) and the imidazolium crosslink, methylglyoxal-lysine dimer (MOLD) (Al-Abed and Bucala, 1995; Ahmed *et al.*, 1997), while its major product is the arginine-derived hydroimidazolone Nδ-(5-hydro-5-methyl4-imidazolon-2-yl)-ornithine (MG-H1) which accounts for 90% of adducts (Biemel *et al*., 2002; Ahmed and Thornalley, 2005; Dobler *et al.,* 2006). The major AGE products of glyoxal are CML (fig. 1.8) and glyoxal-lysine dimer (GOLD) (Odani *et al.,* 1998; Glomb and Monnier, 1995). These AGEs are the major biomarkers of the Maillard reaction in tissue proteins that accumulate in the body with age and in chronic diseases, generating oxidative stress (Ahmed, 1997; Shangari *et al.,* 2004).

Sugars vary in their reactivity in the Maillard reaction, with the rate of AGE formation on intracellular proteins being slowest in the presence of glucose and more rapid with intracellular natural sugars such as fructose, glucose-3-phosphate and glucose-6-phosphate (McPherson *et al.,* 1988). The principal carbohydrate used as an energy source for glycolysis by spermatozoa is fructose, though glucose is thought to also have a role, and both drive hyperactivated sperm motility (Mann, 1946; Williams and Ford, 2001). During metabolism, these sugars could also potentially be the substrates of glycation reactions, and consequently, the source of AGE formation on sperm.

1.4.3 AGEs in male reproduction

Studies have reported the effect of diabetes on standard semen parameters and pregnancy outcome with conflicting results (Mulholland *et al.,* 2011; La Vignera *et al*., 2012; Agbaje *et al.,* 2007). However, there have been consistent reports showing that male diabetics have higher sperm DNA damage, higher ROS levels, and lower antioxidant capacity than non-diabetic subjects (Agbaje *et al.,* 2007). Karimi *et al.* (2011) found that expression levels of RAGE were higher in sperm samples from diabetic patients and this correlated with an increase in sperm DNA fragmentation. It is likely that the AGE-mediated damage arises due to oxidative stress. 8-oxoguanine is a biomarker for oxidative DNA damage and has also been detected at elevated levels in diabetic patients in correlation with DNA fragmentation (Agbaje *et al*., 2008). Sperm DNA damage has previously been associated with lower probability of pregnancy and lower implantation rates (Meseguer *et al.,* 2011). However, the real effects of sperm DNA fragmentation on embryo quality and development are inconclusive and require further investigation (Zini *et al*., 2011).

The principal mode of action of AGE occurs through interaction with the cell surface receptor, RAGE. RAGE belongs to the immunoglobulin (Ig) superfamily and is a pattern recognition receptor which can bind to numerous ligands. The AGE-RAGE pathway has been implicated in a number of diabetic complications (reviewed in Yan *et al.,* 2008). It has also been implicated in adverse birth weight outcomes (Chiavaroli *et al*. 2012). AGE-RAGE signalling activates pro-inflammatory pathways through the transcription factor nuclear factor κB (NF-κB) (Yan *et al.,* 2008; Bierhaus *et al.,* 2001). Once activated, NF-κB binds and activates a number of pro-inflammatory genes including cytokines and adhesion molecules. NF-κB also feeds back to trigger expression of more RAGE and the production of ROS (Yao and Brownlee, 2010). Therefore, activated cells remain viable while also becoming more responsive to AGEs (Bierhaus *et al.,* 2001). RAGE has been detected in cells of the testis, epididymis, and on the sperm acrosome (Mallidis *et al.,* 2007) and a major consequence of AGE-RAGE binding for

sperm is in the generation of ROS through activation of NADPH oxidase (Wautier *et al*., 2001), which can be damaging to sperm due to their vulnerability to oxidative stress.

In vitro glycation of proteins with sugars or AGE intermediates has provided insight into the exact mechanisms of protein modification in terms of which amino acids are targeted and of the functional consequences of glycation. For example, MG and GO inhibit the binding capacity of epidermal growth factor receptor (EGFR) with EGF in a time and dose-dependent manner in cultured cells due to modification of the receptor (Portero-Otin *et al.,* 2002). The impact of glycation on the structure and function of platelet-derived growth factor receptor and insulin have also been illustrated using MG (Cantero *et al.,* 2007; Jia *et al.,* 2006). As AGEs are present on sperm (Mallidis *et al.,* 2007), it is likely that glycation of sperm proteins occurs during spermatogenesis. If this occurs on functionally important proteins, fertilisation capacity could be diminished and could explain the poorer sperm quality in diabetics. As described in section 1.1.4, *Spam1* is a sperm plasma membrane protein with hyaluronidase activity that breaks down HA in the ECM of the cumulus layer surrounding the oocyte and the ZP. *Izumo1* is another sperm membrane protein essential for sperm-egg binding: *IZUMO*-null sperm can penetrate the zona pellucida but cannot bind to the egg (Inoue *et al.,* 2005; Inoue *et al.,* 2013). Finally, Fertilin beta (ADAM2) is one subunit of the Fertilin complex present on the sperm plasma membrane that interacts with integrins on the egg surface through its disintegrin domain during fertilisation (Chen *et al*., 1999). These proteins are indispensable for successful fertilisation and their exposure to glycating agents during spermatogenesis in the testes and in the seminal plasma could contribute to their malfunction.

1.5 Summary

An extensive amount of research on the causes of idiopathic male infertility has been carried out. However, it is still unclear to what extent lifestyle and

environmental factors can modify sperm quality at the physiological, molecular or genetic level.

As some studies have found evidence that certain exposures may be heritable through the male germline, epigenetic changes have been implicated as the primary mechanism. Given the breadth of roles of the epigenome, and specifically of DNA methylation, numerous different pathways could be leading to sperm dysfunction and to adverse ART outcomes. Many studies have focussed on the imprinted genes and on global methylation as the source of this dysfunction, while others have found that candidate non-imprinted genes associated with infertility are affected by lifestyle and environment. Although controlled mouse studies have conclusively shown that lifestyle and environment can affect sperm quality, methylation and pregnancy outcomes, further human studies are needed to determine which, if any, of these factors are true risks.

Obesity, diabetes and ageing have been implicated as drivers of male infertility, yet very little research has been carried out on the role of AGE compounds in disrupting reproductive function. Given that AGEs have a primary role in age- and diabetes-related comorbidities, they could present an important risk to sperm function. *In vitro* cellular glycation experiments have shed light on the effects of glycation in other cell types, however, none so far have done this with sperm. The use of a relatively straight forward *in vitro* glycation assay to highlight the effects of AGEs on sperm could stimulate further research into this new field and help to expose additional mechanisms behind infertility.

Overall, in order to successfully tackle the increasing rates of male infertility, it is important to identify which lifestyle and environmental factors cause changes to sperm function and what the mechanisms behind them are.

1.6 Aims

The aims of this study were to determine whether lifestyle and environmental factors affect sperm quality through the analysis of standard semen parameters and of sperm DNA methylation. We then sought to determine whether these changes to sperm function or DNA quality could have implications for ART outcomes. Using an *in vitro* assay for glycation as a model for ageing and diabetes, we also sought to investigate the action of AGEs in sperm and on functionally relevant sperm proteins.

1.6.1 Objectives:

1. To develop assays for and use bisulphite pyrosequencing to quantify DNA methylation at functionally important DMRs at maternally and paternally imprinted genes and at important non-imprinted genes in sperm. To determine whether this methylation and global methylation levels in sperm are affected by lifestyle and environmental factors, in men attending a fertility clinic, and whether these affect ART outcomes.

2. To determine whether lifestyle and environmental factors are associated with aberrant semen parameters and ART outcomes in men attending a fertility clinic.

3. To develop an *in vitro* glycation assay to investigate the effects of glycation on sperm and sperm DNA damage. To determine whether AGE-modifications affect specific sperm protein function.

Chapter 2

Materials and methods

2.1 Lifestyle, DNA methylation and ART outcomes

Men undergoing assisted reproductive techniques at the department of Reproductive Medicine, Saint Mary's Hospital, Central Manchester NHS Foundation Trust, were recruited for research approved by NRES Committee North West-Preston, REC Reference: 12/NW/0482, ERP/91/078 (Altakroni, 2015) and REC Reference: 14/NS/0082 (appendix 4).

Upon consultation for treatment, men where asked whether they would like to participate in the study by donating the remainder of their sperm sample following treatment, and by completing a questionnaire detailing their health, lifestyle, environmental exposures and diet (Altakroni, 2015; appendix 1). Sperm samples were produced and the questionnaire filled out on the day of egg collection. Figure 2.1 shows the number of sperm samples collected with corresponding questionnaires that were used for DNA methylation analysis and the number of questionnaires that were collected for analysis of lifestyle, sperm parameters and ART outcomes only.

*[*The questionnaire was designed by A. Povey and B. Altakroni from the Institute of Population Health, University of Manchester (Altakroni, 2015)].*

2.1.1 Sample collection

Samples were produced by masturbation after 2-5 days of abstinence as recommended by the hospital. Two sperm samples were collected for each participant on the day of their treatment in sterile plastic containers: one containing neat semen, and the other containing prepared sperm which had undergone density gradient centrifugation in preparation for ART. Semen

analysis was carried out by clinical staff for neat and prepared sperm according to the WHO references guidelines (WHO 5th edition). Prepared sperm was not obtained on all occasions when there was no sample remaining after treatment. Sperm was then transferred from the hospital to the laboratory for DNA analysis. Whole sperm in neat semen samples was separated from seminal plasma by centrifugation at 500 x g for 10 minutes and removal of the plasma supernatant, followed by two washes in 1xPBS at 500 x g for 10 minutes. Prepared sperm samples were washed twice in 1xPBS as above to remove sperm preparation media. Sperm was counted, pelleted and frozen at -80°C until further analysis. Initial samples [91] were collected, processed and stored by B. Altakroni. Subsequent samples [31] were collected, processed and stored by C. Nevin.

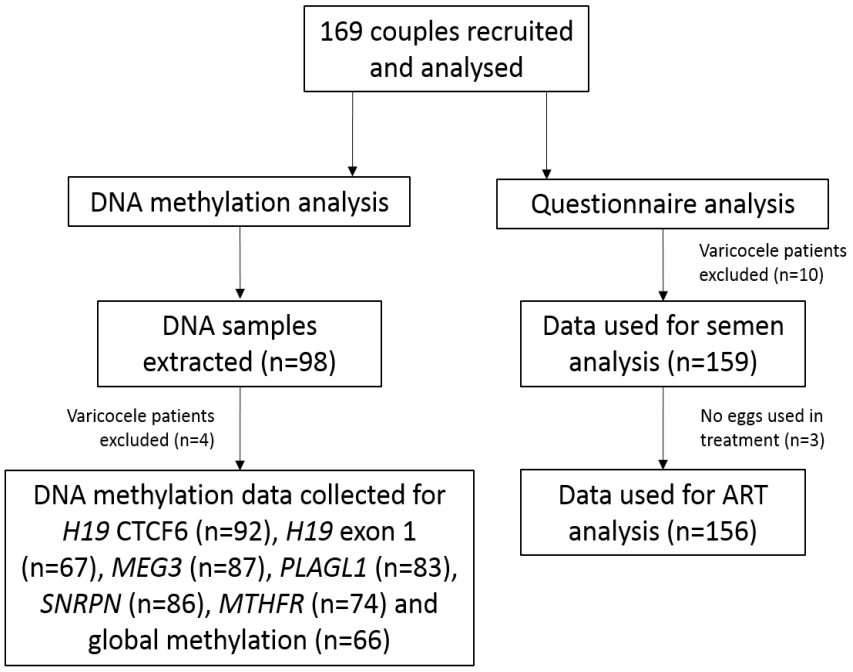


Figure 2.1 Outcomes of recruited participants for DNA methylation analysis and questionnaire analysis

Data on for each participant was collected from the hospital database. Sperm parameters data obtained included semen volume, sperm concentration, % progressively motile and % immotile sperm. IVF and ICSI outcomes data included the number of oocytes used in treatment, the number of fertilised oocytes, the number of cleavage stage embryos, the number of embryos transferred into the female, and the pregnancy outcome (yes or no). Information was also obtained on the hormonal protocol used for oocyte stimulation in the female as well as male and female age. The fertilisation rate (%) was calculated by dividing the number of successfully fertilised oocytes by the number of oocytes used for treatment and the cleavage rate (%) was calculated by dividing the number of cleavage stage embryos by the number of fertilised oocytes.

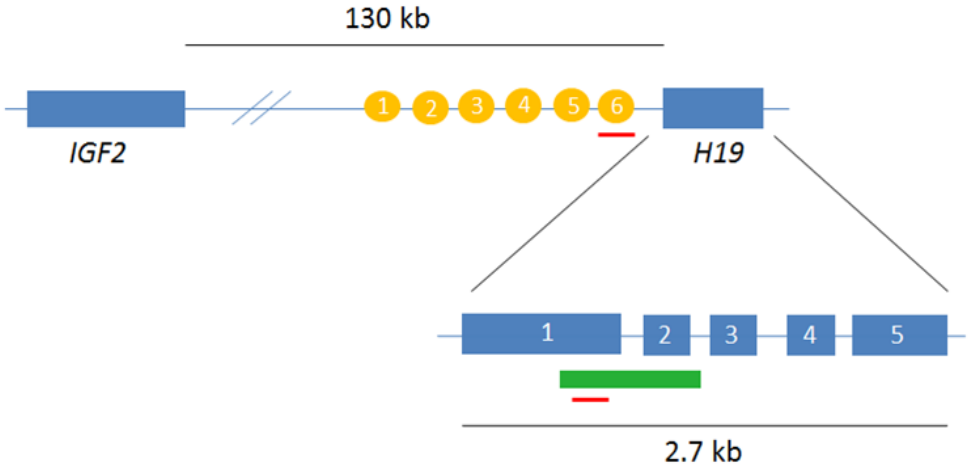


Figure 2.2 Schematic diagram picturing the structure of the *IGF2-H19* locus.

H19 exists 130 kb downstream of *IGF2* on the reverse strand of Chr11. CTCF binding sites are shown as orange circles. A more detailed version of the *H19* gene is shown with exons 1-5 as blue boxes. The green area beneath the 1st and 2nd exons represents a CpG island. Two red lines show the approximate regions that were analysed by pyrosequencing.

2.1.2 Normalisation of semen parameter data

Statistical analysis requires input data to follow a Gaussian, or normal, distribution. When this is not the case, transformations must be applied to the data to reach normality. An initial analysis of histograms and Quantile-Quantile (Q-Q) plots for semen parameters was carried out to check for normality (appendix 6). A histogram illustrates the frequency distribution of the data while Q-Q plots show the observed versus predicted values in the data set: if the data points follow a linear trend in the Q-Q plot (a straight line) then the assumption of normality has been met. A Gaussian distribution in a frequency histogram should show a bell-shaped curve. Shapiro-Wilk values for skewness were also considered for each sample as an approximation of normality but observation of the shape of the data was the ultimate consideration to prevent too many exclusions of samples in the data set.

Data for sperm concentration and sperm count were not normally distributed and had a positive skew. To convert the data to a normal Gaussian distribution the log base 10 function was used in the equation $x' = log_{10}(x)$ where x' is the transformed value, and x is the raw value. The geometric means and 95% confidence intervals (CIs) were calculated by back-transforming the log_{10} values, which consists of taking the anti-log ($log^{x'} = x$). Data for % progressive motility (PM) and % immotile sperm did not require transformation as these showed a normal distribution. Both data sets followed the linear expected values on the Q-Q plots. Semen volume was normally distributed when extreme outliers were removed.

2.2 DNA methylation analysis by pyrosequencing

Quantification of DNA methylation at CpG sites was achieved using bisulphite pyrosequencing. Briefly, genomic DNA was extracted and treated with sodium bisulphite to convert non-methylated cytosine nucleotides to uracil, followed by PCR amplification of the region of interest using primers designed to target the bisulphite DNA. A sequencing primer was designed to target the region of interest and pyrosequencing was carried out using the PyroMark Q24 system (Qiagen, UK).

2.2.1 Pyrosequencing assay design

Regions of interest for each gene were chosen based on current literature which were deemed to be important regulatory regions for DNA methylation. Gene structure was observed on human genome assembly GRCh37/hg19 using the UCSC genome browser (Genome Biolinformatics Groups of UC Santa Cruz, 2015). Regions of interest were determined by looking at the presence of CpG islands, and for promoters, the histone acetylated lysine mark (H3K27Ac) which is found near regulatory regions and thought to enhance transcription (Shyueva *et al.,* 2014). If no CpG islands were detected in the region of interest, for example with *MEG3* intergenic- (IG-)

DMR, sequences were input to EMBOSS CpG predictor software (Genome Bioinformatics Groups of UC Santa Cruz) and the minimum CpG length was set to 100 bp; primers were then designed for within or near these regions. Primer sequences for each region were either purchased as pre-designed CpG assays from Qiagen UK (table 2.1), or custom oligonucleotides were designed using the PyroMark Assay Design software (Qiagen, UK) and purchased through Life Technologies (UK) (table 2.2). Sites analysed included the *H19* exon 1-spanning CpG island and the 6th CTCF binding site of the upstream ICR which controls *H19* and *IGF2* expression (fig. 2.2). Targets for analysis included the ICR between *MEG3* and *DLK1,* the *PLAGL1* CpG island spanning the first exon, the upstream region of the *SNRPN* CpG island, and the promoter regions of *MTHFR, DAZL* and *NR3C1* promoters (fig 2.3). Chromosomal locations and the number of CpG sites analysed are given in tables 2.2 along with the sequence analysed for the pre-designed assays (table 2.1).

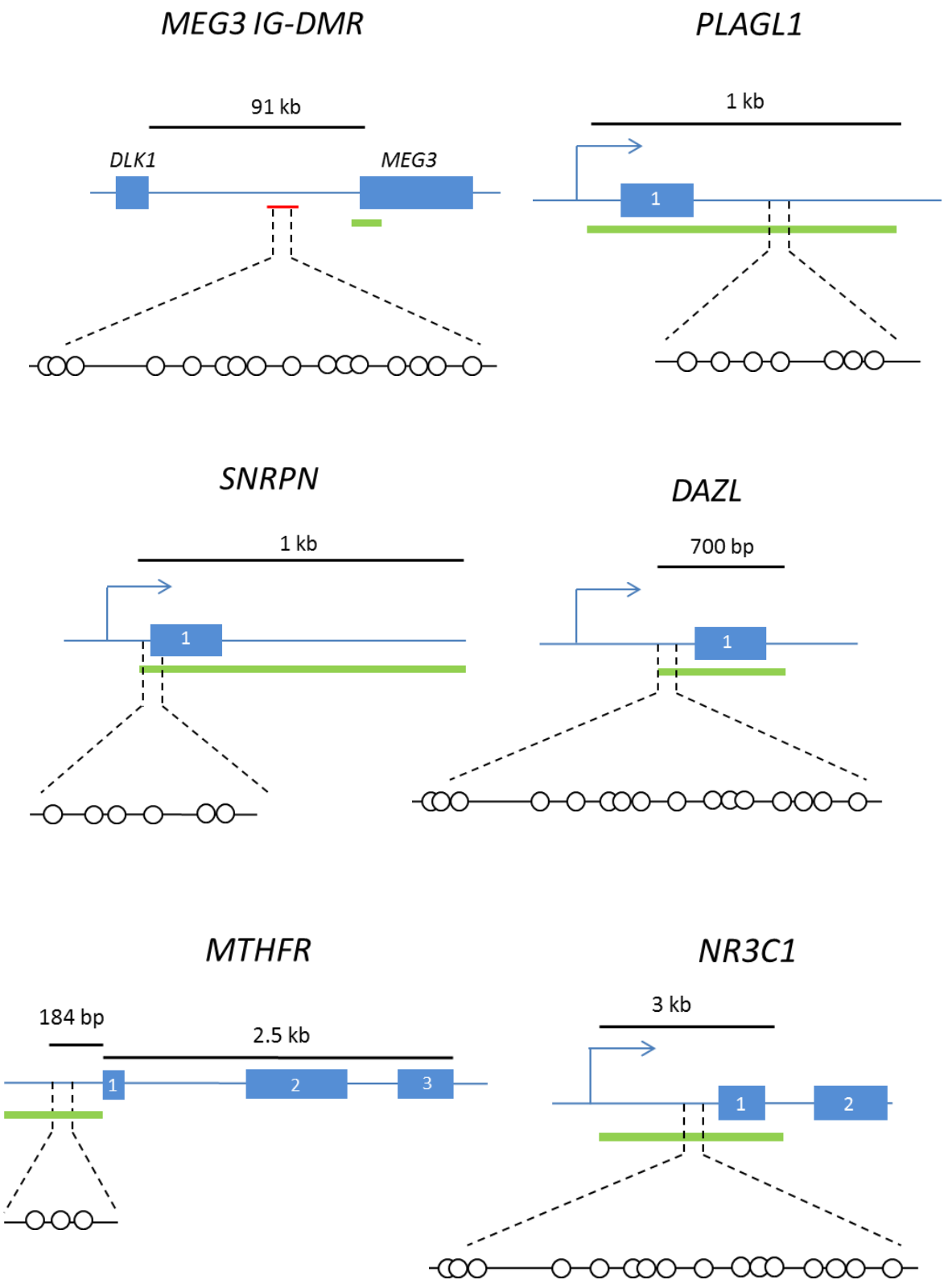


Figure 2.3 Gene schematics

Blue boxes represent individual exons for *PLAGL1, SNRPN, DAZL, MTHFR, NR3C1* and whole genes for *MEG3* IG-DMR. Green bars represent CpG islands. White circles show the number and distribution of CpG sites analysed. Black bars show approximate genomic distances.

2.2.2 Sperm DNA extraction for bisulphite pyrosequencing

DNA was extracted from up to $25x10^6$ sperm using the Qiagen Blood and Tissue Midi Kit (Qiagen, UK). Kit components contained the following: G2 (lysis) buffer, QC (wash) buffer, QBT (equilibriation) buffer, QF (elution) buffer and filter columns. Consistent methods were used for DNA extractions carried out by B. Altakroni and on later samples by C. Nevin.

Sperm pellets were resuspended in 1 ml of 1xPBS, and were added to a falcon tube containing 9.5 ml of G2 buffer with 19 µl RNase (10 mg/ml) (Sigma, UK) and 250 µl Proteinase K (1 mg/ml) (Sigma, UK). The tubes were incubated at 4°C overnight on a rotator. Next day, 0.5 ml dithiothreitol (DTT) (5 mM) (Sigma, UK) was added to the tubes and incubated at 4°C for 1 hour on a rotator (PODC Scientific, UK). The tubes were then transferred to a 37°C water bath for 1 hour. A sufficient number of Qiagen filter columns were set up and equilibriated with 4ml of QBT buffer. The entire contents of the samples were then added to the columns which passed through by gravity flow. The columns were then washed twice with 7.5 ml QC buffer. DNA was eluted from the columns with 4 ml QF buffer and the flow through was collected. DNA was precipitated by adding 2.8 ml room temperature isopropanol (Fisher, UK) to each collection tube and inverting several times. Samples were centrifuged 12,000xg for 15 minutes at 4°C. Isopropanol was removed and 1 ml of 70% ethanol (Sigma, UK) was added to the pellet, resuspended and centrifuged 12,000xg for 5 minutes at 4°C. Ethanol was removed and the pellet was spun again for 2 minutes. All remaining ethanol was removed and pellets were allowed to air dry from approximately 2 minutes before dissolving in 50-100 µl of TE buffer (pH 7.5). DNA was quantified using a Synergene HT nucleic acid plate (Biotek, UK).

2.2.2 Bisulphite conversion

DNA methylation involves the chemical modification of cytosine nucleotides by the addition of a methyl group. When treated with sodium bisulphite, unmethylated cytosine residues undergo deamination to uracil, while

methylated cytosine (5mC) remains as cytosine (fig. 2.4). Uracils are subsequently converted to thymine during PCR amplification while the methylated cytosines are amplified as cytosines. Subsequent sequencing of the PCR product allows the discerning of the methylation status of each CpG site.

Sperm DNA was diluted to 100 ng/µl in nuclease-free water and 500 ng was used for bisulphite conversion with the EpiTect Fast DNA Bisulphite Kit (Qiagen, UK). According to the manufacturer's instructions, 500 ng DNA was added to reaction tubes containing 80 µl bisulphite solution and 15 µl DNA protect buffer, to a final volume of 140 µl. The PCR tubes were placed in a thermal cycler (Thermo Scientific) and bisulphite conversion was completed using the programme detailed in table 2.3.

Upon completion, bisulphite converted DNA was transferred to sterile 1.5 ml Eppendorf tubes and 310 µl of buffer BL was added and vortexed to mix. 250 µl of ethanol (100%) was then added and again pulse vortexed to mix. The contents of each tube was transferred to a corresponding EpiTect Fast DNA Bisulphite Kit spin column for clean-up. Columns were centrifuged at maximum speed and washed once with 500 µl buffer BW followed by incubation of the tubes with 500 µl buffer BD for 15 minutes at room temperature. Spin columns were centrifuged, flow-through discarded, and two washes with 500 µl buffer BW were performed, followed by one wash with 250 µl ethanol (100%). One further spin was carried out to remove residual liquid and columns were incubated in a heat block for 5 minutes at 60°C to evaporate remaining liquid. Following a 1 minute incubation, DNA was eluted from the columns in 15 µl buffer EB. Bisulphite DNA concentration was estimated using the Nanodrop plate reader (Thermo-Fisher Scientific, UK) under single-stranded DNA settings and diluted to 5 ng/µl in TE buffer. Bisulphite DNA was stored at -20°C.

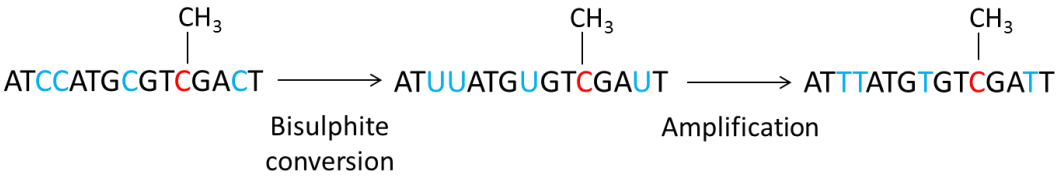


Figure 2.4 Bisulphite conversion of genomic DNA and PCR amplification

Methylated cytosine nucleotides present in CpG sites (red) remain as cytosines while non-methylated cytosines (blue) within and outside CpG sites are converted to uracil and subsequently replaced by tyrosine during PCR.

Table 2.1 Pre-designed Qiagen CpG assays for pyrosequencing. All biotin modifications were on the reverse primer

Gene	Assay name Catalogue no. Chromosome location	Amplicon length (bp)	Sequence to analyse	CpGs sites
H19 exon 1	Hs_hsa_mir_675_01_PM PM00046823 Chr11:2017874-2018037	163	GCATGGGGCGAGACCAGACTAGGCGAGGCGGGCGGGGCGGA	5
PLAGL1	Hs_PLAGL1_02_PM PM00122087 Chr6:144385071-144385295	224	GCGCCCGCCCCGCCGGCCACTGTCCCCGCGCGGA	7
MTHFR	Hs_CLCN6_01_PM PM00000091 Chr1:11865545-11865777	232	GGTCACTGAGTCACCGATGGGGGCGAGGAYACGGGC	3
PEG10	Hs_SGCE_03_PM PM00030212 Chr7:94286100-94286304	204	CGCTTCACGCGGGCGA	4
SNRPN	Hs_SNURF/SNRPN_01_PM PM00168252 Chr15:25200006-25200255	249	GCACGCCTGCGCGGCCGCAGAGGCAGGCTGGCGCGC	6
CD247	Hs_CD247_01_PM PM00095452 Chr1:167408761-167408986	225	TCTGCGCACGCGCGAGGGCGCTAGCCCGGGA	6

Table 2.2 Pyrosequencing custom oligonucleotides

Gene	Chromosome location	Forward primer / Reverse primer (biotin) / Sequencing primer	CpG sites
DAZL	Chr3: 16647040-16647131	GTGTGTTTGTGGGTTTATGTGA	16
		CACCACTTCTAAAACTACTATAAAATC	
		TGTGGGTTTATGTGAG	
MEG3	Chr14: 101250091-101250246	TAGAGTAGGTTTGAGAGAGTGT	12
		ACATCATACAACCTAAACTTTCTACAA	
		TTGAGGAGGGGAGTT	
NR3C1	Chr5: 142783597-142783677	AGGAAGAGGGAGAGATAGTATGGT	7
		AAACACACACATCCCCACAT	
		AGGGAGAGATAGTATGGTG	
H19 CTCF6	Chr11: 2019589-2019737	TTTGGGAGAGTTTGTGAGGT	13
		CCCCCAAACCCATTCCCATCCAATTA	
		GTTTATAGTTTGTTAGTAGAGTG	
LSP1	Chr11:1881555-1882154	AGGAAGAGGGAGAGATAGTATGGT	4
		AAACACACACATCCCCACAT	
		AGGGAGAGATAGTATGGTG	

2.2.4 Amplification of bisulphite DNA

Pyrosequencing target regions were amplified using the PyroMark PCR Kit (Qiagen, UK). 25 µl PCR reaction tubes were set up according to table 2.4. Gradient PCR was initially carried out to determine the optimal annealing temperature for amplification ranging from 52-60°C and cycling conditions were carried out according to table 2.5. 95°C for 15 minutes; followed by 45 cycles of denaturation, 94°C for 30 seconds; annealing, 56°C for 30 seconds; extension, 72°C for 30 seconds. Finally, an extension of 72°C for 10 minutes was included. Gradient PCR products were run on a 3% agarose gel containing Midori Green (0.005%) in TBE buffer at 80 V for 30 minutes and observed under a UV transilluminator (GBOX) for visualisation of bands. PCR products were frozen at -20°C until pyrosequencing.

Table 2.3 Cycling conditions for bisulphite conversion.

Step	Temperature (°C)	Duration (minutes)
Denaturation	95	5
Incubation	60	10
Denaturation	95	5
Incubation	60	10
Incubation	20	Hold

2.2.5 Pyrosequencing

Bisulphite pyrosequencing was used to quantify the mean methylation of single CpG sites in sperm samples. This method is a sequencing-by-synthesis process in which the nucleotides, adenine (A), cytosine (C), thymine (T) and guanine (G), are sequentially added to a known DNA

sequence and nucleotides that are complementary to the DNA template are incorporated by DNA polymerase (fig. 2.5). Each time a nucleotide is incorporated to the sequence a molecule of pyrophosphate (PPi) is released which is converted to ATP by ATP sulfurylase. This ATP drives the conversion of luciferin to oxyluciferin by luciferase, resulting in the release of visible light which is detected by the pyrosequencer and appears as a peak on the resulting pyrogram. The release of light is directly proportional to the number of nucleotides incorporated. For example, if two guanine nucleotides are incorporated at once, the peak height will be twice that of when one guanine nucleotide is incorporated. The sequential incorporation of nucleotides allows the determination of the sequence.

Pyrosequencing was carried out using the PyroMark Q24 and Q24 Advanced software and kits. Firstly, DNA was bound to Streptavidin Sepharose High Performance beads (GE Healthcare, UK). A master mix was made up for the required number of samples to be run with each sample containing 1 µl of beads, 40 µl binding buffer and 29 µl water. 70 µl master mix was transferred to a 0.2 ml PCR tube and 10 µl bisulphite DNA (5 ng/µl) was added bringing the total volume to 80 µl. Tubes were incubated on a shaker at maximum speed for 10 minutes at room temperature. Meanwhile, the sequencing primer was prepared. Firstly, a new assay was generated on the PyroMark Q24 software for each specific site and the sequence to analyse was input and a nucleotide dispensation order was produced. The required assay was

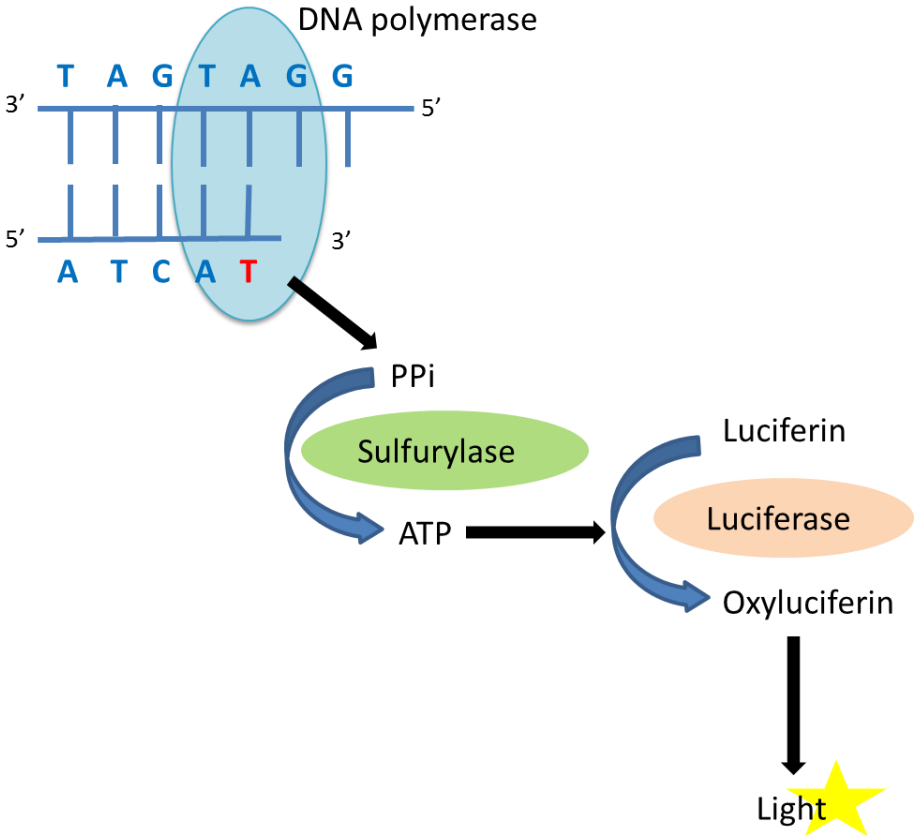


Figure 2.5 The pyrosequencing reaction

The incorporation of a nucleotide into the sequence by DNA polymerase results in the emission of light through a series of reactions. The amount of light emitted is proportional to the number of nucleotides incorporated.

selected for each run along with the number of samples in the plate which gave the volumes of Enzyme and Substrate mixtures and of dNTPs to load into the cartridge (Qiagen, UK) based on the length of the assay sequence. The cartridge was loaded and secured in place in the Pyrosequencer. Sequencing primers were either diluted to 1X if using pre-designed CpG assays or to 0.3 µM for custom primers in annealing buffer (Qiagen, UK) and 25 µl was dispensed into each well of a 24-well PyroMark sequencing plate (Qiagen, UK). The PyroMark Q24 workstation was then run: using a vacuum tool, DNA-bound beads were sequentially washed in 70% ethanol (Sigma, UK), denaturing buffer (Qiagen, UK), annealing buffer and wash buffer (Qiagen, UK). Beads were then transferred to the sequencing plate containing the sequencing primer. The plate was immediately transferred to a heat block set to 80°C and incubated for 2 minutes. Following this, the plate was secured into the Pyrosequencer and the sequencing experiment was run. Pyrosequencing output was in the form of a pyrogram (fig. 2.6) and results were analysed using the PyroMark Q24 software.

PyroMark Q24 software gave an accuracy reading for each of the CpG sites analysed based on how correct the nucleotides in the surrounding sequence had been sequenced: red (failed), yellow (check), blue (passed). Red reads were repeated or excluded from the analysis.

Table 2.4 Reaction mix for PyroMark PCR

	Final concentration	Volume (µl)
PCR Master Mix	1x	12.5
CoralLoad	1x	2.5
Forward primer	0.3 µM	0.75
Reverse primer	0.3 µM	0.75
DNA	5 ng/µl	2
NF-H_2O	--	6.5
Final volume		25

Table 2.5 PyroMark PCR cycling conditions

Step	Temperature (°C)	Duration	Cycles
Initial denaturation	95	15 s	1
Denaturation	94	30 s	
Annealing	Variable	30 s	45
Extension	72	30 s	
Final extension	72	10 min	1
Storage	4	Hold	

2.2.6 Intra-individual variability (inter- and intra-day variability)

Coefficient of variation (%CV) was calculated to determine the variation between pyrosequencing reads between samples obtained from the same PCR product and those obtained from different PCR products. %CV was calculated as ((SD/mean)*100). The mean %CV for the same PCR products run on the same sequencing plate was 1.69%. The mean variation for the same PCR products sequenced on different sequencing plates was 3.93% and the mean variation for different PCR products sequenced on the same sequencing plate was 2.74%. The hypomethylated genes, *PEG10*, *SNRPN*, *PLAGL1, DAZL* and *MTHFR* were not used to calculate the %CV as their extremely low methylation values caused huge changes to the %CV values when there were very small changes, i.e. a 1% change from a gene that is 5% methylated would generate a %CV much greater than a 1% change in a gene that is 90% methylated.

2.3 Global DNA methylation ELISA

Global DNA methylation was measured by the concentration of 5-methyl-cytosine (5MedCyd) using the Global DNA Methylation ELISA Kit (Cambridge Biosciences, UK). Kit components included a 96-well 5MedCyd DNA conjugate plate, anti-5MedCyd antibody, secondary HRP-conjugated antibody, assay diluent, 10x wash buffer, substrate solution, stop solution and 5MedCyd standard.

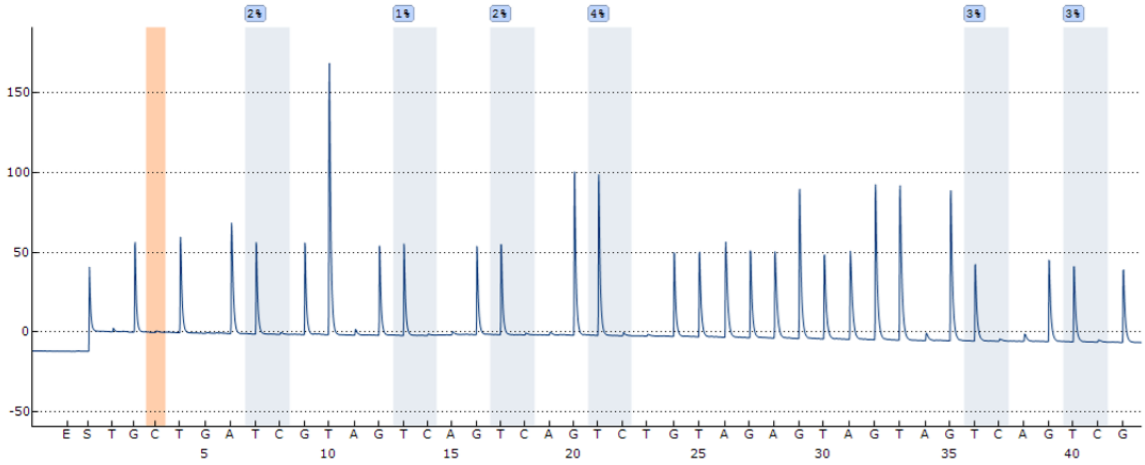


Figure 2.6 The pyrosequencing output: pyrogram

Peak height in the pyrogram correspond to the number of nucleotides incorporated. A negative control is included (orange line) which shows no peak. Blue boxes show the methylation reads (%) correspond to the percentage of methylated sites in the sample.

2.3.1 Preparation of DNA

500 ng of genomic DNA which was extracted using the above method (section 2.2.2), was converted to single-stranded DNA by incubating at 95°C for 5 minutes and chilling on ice. DNA was then digested into nucleosides by incubating with 5 units of nuclease P1 (Sigma, UK) for 2 hours at 37°C, followed by treatment with alkaline phosphatase (Sigma, UK) for 1 hour at

37°C in 100 mM Tris, pH 7.5. The reaction mixture was centrifuged for 5 minutes at 6000xg and the supernatant was used for the ELISA assay.

2.3.2 Preparation of conjugate ELISA plate

Standards of 5MedCyd were prepared from a stock solution provided in the kit ranging from 0-10 µM in assay diluent. The conjugate plate was rehydrated in assay diluent for 1 hour at room temperature on an orbital shaker and washed once in 1xPBS. 50 µl of prepared DNA sample or 5MedCyd standard was added to the wells and incubated for 10 minutes on an orbital shaker. All standards were assayed in duplicate while five DNA samples were assayed in duplicate. 50 µl of anti-5MedCyd antibody was then added to each well, mixed and incubated for 2 hours at room temperature on an orbital shaker. Following incubation, wells were washed four times in 1x wash buffer and 100 µl secondary HRP-conjugated antibody was added to each well and incubated at room temperature for 1 hour on an orbital shaker. Wells were again washed four times in 1x wash buffer. For the colorimetric detection, 100 µl substrate solution was added to all wells and incubated on an orbital shaker until a distinct colour range appeared across the standards. The reaction was then stopped by adding 100 µl stop solution to each well. Absorbance was read at a wavelength of 450 nm on a Synergene HT plate reader (Biotek, UK).

2.4 Health and lifestyle questionnaire

Participants completed a questionnaire consisting of two sections (appendix 1): in the first section they were asked to report their exposure to smoke, attendance at work and diet in the last 24 hours. In the second part, participants were asked about their occupational exposures and hazards, their exposure to chemical or physical agents at home or in their job, their health, lifestyle and finally a food frequency questionnaire. This questionnaire

was designed for a previous study investigating male infertility (Altakroni, 2015) and was implemented here as a large source of information relevant to DNA methylation analysis.

2.4.1 Categorisation of health and lifestyle data

In the health section of the questionnaire, all answers were put on a binary scale, “0” or “1”. Having fever or flu, or job illness was recorded as binary and the duration was not considered. Participants were asked about job-related illnesses; having stress, depression or anxiety was the only variable included in subsequent analyses due to its previous association with effects on sperm, while physical injury (e.g. breathing or lung problems) were not. Of those participants who self-reported sleep problems, most of these also confirmed to suffer from stress, therefore these two variables were combined under the single variable of “stress”. Type of underwear worn was recorded as either wearing boxer shorts or not wearing boxer shorts.

Smoking habit was investigated on a binary scale as either non- or ex-smokers as no participants reported to be current smokers, and also on a continuous scale as the number of years the participant had smoked. Smoking duration (years) was calculated from the difference in the reported age of starting and stopping smoking. The type of cigarettes smoked was not included in the analysis. Alcohol consumption was similarly investigated on a binary scale as those who drink <1 drink per week or ≥1 drink per week, and on a continuous scale as units consumed per week. Participants recorded the type and number of alcoholic beverages they consumed per week and units of alcohol per drink were recorded as: one pint of 4% beer, 2.3 units; one pint of 4.5% lager or cider, 2.6 units; one 175ml glass of 13% wine, 2.3 units; one 25 ml measure of 40% spirits, 1 unit (Drinkaware, 2016).

To determine physical activity levels, participants were asked how many times a week they participated in mild, moderate and strenuous physical activity. The values given for moderate and strenuous exercise were then

input to the Godin equation which gave a leisure score index (LSI) for each participant: $LSI = (5 * moderate) + (3 * strenuous)$. Participants that scored ≤23 were classed as insufficiently active and those that scored ≥24 were classed as active based on previous reports (Amireault and Godin, 2015).

Participants were asked about their exposure to a number of hazards either at work or at home, and answered as “yes” or “no”. Factors which had less than 10% of participants either exposed or not exposed were excluded from further analysis due to the possibility for low numbers to give false positive results.

2.4.2 Food frequency questionnaire

Men were asked how regularly they consumed seventy-eight food items on a scale of 1-7 for 1) never, 2) less than once a month, 3) once or twice a month, 4) once per week, 5) 2-3 times per week, 6) 4-6 times per week and 7) every day. These food items were then grouped into 28 categories and the mean frequency of consumption was calculated for all the foods in each category. This data was analysed by principal components analysis (PCA) to identify where the greatest variation existed in diets of the study population based on foods consumed. PCA revealed that there were two distinct diet types with the greatest amount of variation found in the first and second principal components; these were diets generally deemed to be “healthy” and “unhealthy”. The foods that correlated together in the healthy diet included vegetables, nuts, cereals, green tea, vitamins, soy and low fat dairy, compared with the unhealthy diet in which foods that correlated included red, fried and processed meat, fries, butter, high fat dairy, bread, sugar and confectionary, and caffeinated sugar sweetened beverages (Coca-Cola, Pepsi, Red Bull). Items excluded from the analysis were food wrapped in cling-film, tinned food and spicy food as their contribution to either of these diets was ambiguous.

Each food group had a proportional loading value in each diet, which can be explained as the relative contribution of that food to the variation in that diet, e.g. red meat has a high loading in the unhealthy diet (0.33) and a very low loading in the healthy diet (0.08), while nuts have a high loading in the healthy diet (0.33) and a low loading in the unhealthy diet (0.03). Food groups which had a loading >0.1 in one diet and <0.1 loading in the other diet were classed as being associated with the former diet. Food groups with loading values <0.1 in both diets were excluded as these contributed very little to the variation in both diets; these included eggs and caffeinated non-sugar sweetened beverages (tea and coffee). In addition, food groups that had high (>0.1) loading values in both diets were excluded as they contributed equally to both diets, i.e. participants that had a high “healthy” score consumed foods that were also consumed by participants that had a high “unhealthy” score. These items included chicken/poultry, liver, fish, fried fish, vegetable oil, fruit, and whole grains.

Healthy and unhealthy diet scores were calculated for each participant by multiplying the frequency of consumption of each food group by the food’s loading value and taking the sum of these scores for each item. Men were then classified into quartiles for each diet type: quartiles for unhealthy diet were <3.975, 3.9751-4.885, 4.8851-5.82, ≥5.821. Quartiles for healthy diet were ≤1.47, 1.471-2.465, 2.4651-3.4025, ≥3.4025.

2.4.3 Statistical analysis

Statistical analysis was carried out using the IBM SPSS Statistics 20 package while graphs were generated using GraphPad Prism 6 software. Descriptive statistics were analysed for each independent and dependent variable, observing means and standard deviations (SD) for raw methylation and semen parameters data, and geometric means and 95% CI when means had been obtained from transformed data.

Differences in all continuous dependent variables (DV) including % methylation, semen parameters, fertilisation and cleavage rates between binary independent variables (IV) (e.g. non-/ex-smoker) were analysed using an independent t-test. Differences in continuous DVs between IVs with three or more categories (e.g. diet quartiles) were analysed by one-way ANOVA and followed up using Tukey's post-hoc test to identify differences between specific groups. Tests were carried out under the assumption of equal variance; when Levene's test for equality of variances was not met (p<0.05), results were taken from the t-test not assuming equal variance. Differences in binary DVs (e.g. pregnancy outcome) between continuous IVs were analysed with non-parametric Mann-Whitney U test, and for binary DVs between categorical IVs the Chi squared (χ^2) test was used. Multiple regression analysis was carried out to determine effects of significant IVs whilst controlling for other variables. IVs that showed multi-collinearity were not included in the same regressions to prevent redundancy. Spearman's and Pearson's correlations were used to identify linear relationship between continuous IV and DVs.

DNA methylation data was transformed according to section 2.1.2. Genes that underwent transformation to normality were *SNRPN, MEG3, NR3C1* and *DAZL* (appendix 12, figures 1-4).

2.5 An *in vitro* sperm glycation assay

To investigate the effects of glycation on human sperm, an *in vitro* model was developed in which sperm were incubated with various glycating agents and formation of a major AGE, CML, was measured. The effects of this glycation on sperm motility, oxidative DNA damage, intracellular ROS levels and HA binding were also measured.

2.5.1 Procurement of semen

Semen samples were obtained from consenting donors (aged 19-51 years), covered by faculty ethical approval at Manchester Metropolitan University (appendix 7). Donors were verbally informed of the study details and given a participant information sheet (appendix 8). They then completed a medical screening questionnaire (appendix 9) and finally signed the participant consent form (appendix 10). Semen was produced on site in a designated, secured room. Participants were instructed how to produce the sample and given a sample collection pot (Sterilin, UK). Participants subsequently signed a semen procurement form each time they donated a sperm sample (appendix 11).

All neat semen samples underwent standard semen analysis according to the WHO 5th edition before processing. Firstly, semen was liquefied at 37°C for 30 minutes. The total volume of semen was then measured and recorded. To measure sperm motility and concentration, 5 µl of semen was applied to a cell counting slide (Vitrolife, UK) and analysed by a Computer Assisted Sperm Analyser (CASA – Sperminator®, Procreative). Depending on downstream experiments, semen samples were then either washed or prepared by density gradient centrifugation as described below.

2.5.2 Washing of sperm and preparation by density gradient centrifugation

Liquefied semen was centrifuged 800 x g for 10 minutes to separate sperm cells from seminal plasma. Seminal plasma was removed and flash frozen in liquid nitrogen before storage at -80C. Some sperm pellets were frozen at this point at -80°C until use. Thawed sperm pellets or fresh sperm pellets were resuspended in 3 ml of Sperm Preparation Media™ (SPM) (Origio, Denmark) and centrifuged 300 x g for 10 minutes. This wash step was repeated once more and cells were resuspended in 1 ml SPM or PBS and counted.

Alternatively, motile sperm were separated from immotile and immature sperm and from non-sperm cells by density gradient centrifugation. Briefly, up to 1 ml of neat semen was layered on top of a gradient of 55% and 80% SupraSperm™ media (Origio, Denmark) and centrifuged 300 x g for 20 minutes. The supernatant was removed and discarded and the sperm pellet was washed twice in SPM (Origio, Denmark), centrifuging at 300 x g for 5 minutes each. The cells were then resuspended in 1 ml SPM and counted.

2.5.3 *In vitro* glycation of sperm

Motile sperm that had been separated by density gradient centrifugation were pelleted and resuspended in solutions of SPM containing either D-glucose (10 mM, 30 mM or 50 mM), methylglyoxal (MG) (50 µM) or glyoxal (GO) (50 µM) (Sigma, UK) at a concentration of $20x10^6$ cells/ml. Sperm were incubated at 34°C on a rotator for 6 days before analysis for CML formation and oxidative DNA damage. Sperm were also glycated for up to 4 hours with cells removed at 0, 2 and 4 hour time points for analysis of motility, hyaluronan binding capacity and CML formation.

2.5.4 MG toxicity assay

The concentration of MG and GO to use in the experiments was determined using a toxicity assay in which sperm were incubated with concentrations of MG at 0, 50, 100, 250 and 500 µM in SPM for 48 hours at 34°C before a vitality stain was carried out to determine the percentage of live sperm.

2.3.4.1 Vitality staining

The one step eosin-nigrosin stain for sperm vitality was prepared based on the guidelines published in WHO 5^{th} edition. The stain is dye-exclusion mechanism in which dead membrane-damaged sperm are stained pink or purple, while live membrane-intact sperm exclude the dye and appear white.

Briefly, eosin Y (0.67%) (Sigma, UK) and sodium chloride (NaCl) (0.9%) (Sigma, UK) were dissolved in water with gentle heating, before nigrosin (10%) (Sigma, UK) was added, and the solution was boiled, then allowed to cool and filtered. Equal volumes of eosin-nigrosin stain and resuspended sperm sample were mixed and smeared onto a microscope slide using the feathering technique. A second smear was made using a fresh aliquot of sperm. Using a light microscope, 200 sperm were counted and the number of live and dead sperm were recorded. % live sperm were calculated as $((\#live\ sperm/\#total) * 100)$.

2.5.6 Measurement of CML by flow cytometry

Sperm were removed from the 6 hour glycation experiments at 0, 2 and 4 hours, and pelleted by centrifugation at 350 x g to remove glycation media. Cells were resuspended in paraformaldehyde (4%) (Sigma, UK) and incubated at room temperature on an eppendorf rotator for 20 minutes. Cells were pelleted again and permeabilised by resuspension in 0.1% PBS-tween for 15 minutes. Sperm were then blocked in 10% normal goat serum (Vector laboratories, UK) in 1xPBS for 1 hour at room temperature. Cells were incubated with a mouse anti-CML primary antibody (1:50) (Life technologies UK) for one hour at room temperature, followed by a goat anti-mouse Alexa Fluor 488 secondary antibody (1:2000) (Abcam, UK) for 45 minutes at room temperature in the dark. A control in which primary antibody was omitted from the procedure was also prepared under the same conditions. Cells were then washed once and resuspended in 1xPBS before acquisition and analysis using a Facs Calibur flow cytometer (BD Biosciences, UK). Cells were gated using the forward-scatter/side-scatter (FSC/SSC) channels recorded in a dot plot, to exclude debris and large cells. Fluorescence was measured in the FL-1 channel and CML levels were recorded as the mean fluorescence intensity (MFI) of cells and recorded on a histogram. The boundary of fluorescence-negative and fluorescence-positive cells was set by gating 1-1.5% of unstained cells in the positive region.

2.5.7 Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) in sperm treated with either glucose, fructose, mannitol, MG or GO was measured using the CM-H2DCFDA General Oxidative Stress Indicator (Invitrogen, UK). CM-H2DCFDA diffuses into cells where it is oxidised to yield a fluorescent adduct that is trapped inside the cell and its emission can be measured in the Fluorescein spectrum. Briefly, treated sperm were washed in pre-warmed PBS and resuspended in PBS containing the ROS probe at 5 µM. The cells were incubated at 34°C for 30 minutes. The dye was then removed by centrifugation and the cells resuspended in PBS and fluorescent intensity was detected in the FITC emission range of the BD Facs Calibur (BD Biosciences, UK). A negative control of sperm unexposed to the dye was made and a positive control of sperm exposed to H_2O_2 for 15 minutes prior to the assay.

2.5.8 Measurement of 8-oxoguanine by flow cytometry

The level of oxidative DNA damage in spermatozoa incubated with glycating agents over 6 days was determined using the fluorometric OxyDNA Assay Kit (Calbiochem®, EMD Millipore, US). The assay is based on a FITC-conjugate that binds to the 8-oxoguanine moiety of 8-oxoguanosine of oxidized DNA. Sperm cells were centrifuged 300 x g for 7 minutes to pellet the cells and remove media. Cells were fixed in 4% PFA and permeabilised with 70% ethanol. Cells were washed with Wash Solution (1x) and resuspended in 100 µL FITC-conjugate (1x), before incubation in the dark for 1 hour at room temperature. Cells were washed once in Wash Solution (1x), resuspended in 1xPBS and analysed by flow cytometry. Flow cytometry was performed as described above, also using the FL-1 channel to determine fluorescence at excitation wavelength of 495 nm.

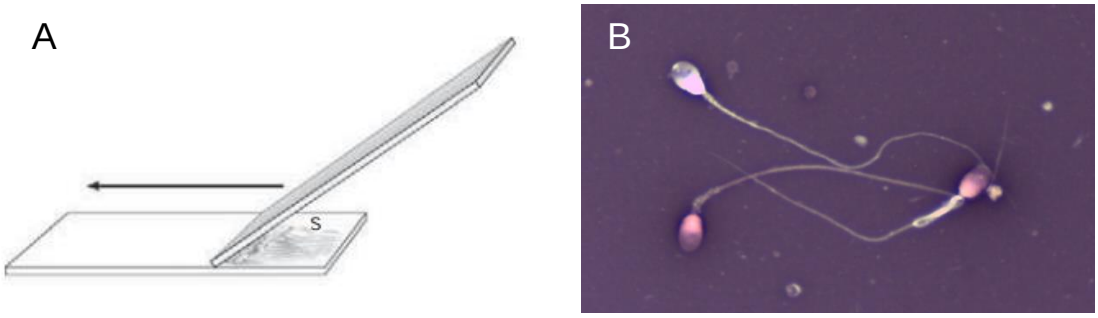


Figure 2.7 Slide preparation of sperm for vitality staining

Sperm vitality was determined using the eosin-nigrosin stain. Stained sperm were smeared onto a glass slide by feathering (A) and visualised as live (white) and dead (pink) by light microscope analysis (B).

2.5.9 Immunocytochemical staining of sperm for AGEs and CML

Glycated sperm were stained for AGEs using a general anti-AGE antibody (Abcam, UK) and specifically for CML using an anti-CML antibody (Abcam, UK). 10 µl of sperm was placed on one end of a glass microscope slide and smeared across the slide using the feathering technique and allowed to air dry. Slides were then fixed by submersion in ice-cold methanol (100%) for 15 minutes. Once dry, a water-resistant pen (Life technologies, UK) was used to seal the area around the cells. Slides were rehydrated in PBS-tween (PBS-T) for 3 minutes before blocking in 10% normal goat serum in PBS-T (0.05%) for 1 hour at room temperature. Slides were washed in 3x fresh changes of PBS-T for 1 minute each. Primary antibodies (anti-AGE or anti-CML) were diluted in PBS-T and added to the slides (1:200) and incubated in a humidified chamber overnight at 4°C. Negative controls were included in which the primary antibody was omitted. Next day, slides were washed in 3x changes of PBS-T and secondary goat anti-mouse (1:2000) or goat anti-rabbit (1:2000) antibodies (Life Technologies, UK) were added to the slides staining for CML and AGEs, respectively. Slides were incubated with secondary antibodies for 1 hour at room temperature in a darkened humidified chamber. Slides were washed a final 3 times in PBS-T before

excess reagent was drained off and sperm nuclei were stained for using Vectashield Mounting Medium with DAPI (Vectashield, UK) and finally coverslipped. Fluorescent images were taken using an Axio Imager Z1 (Zeiss, HBO 100 mercury lamp) with AxioVision 4 software. Sperm head fluorescence was quantified using ImageJ software and the Corrected Total Cell Fluorescence (CCF) was obtained using the equation CCF = initial cell density – (cell area x background intensity).

2.5.10 Sperm hyaluronan binding assay

Functional activity of plasma membrane hyaluronidase in glycated and non-glycated sperm was measured using Hyaluronan Binding Assay (HBA) slides (Origio, Denmark). At 0, 2 and 4 hours of incubation with MG, GO or SPM, 8 µL of sperm suspension was removed and placed onto a hyaluronan-coated slide and a coverslip applied. After a 10 minute incubation period in which sperm were allowed to bind to hyaluronan, the number of bound motile sperm and the number of unbound motile sperm on a 10x10 square grid were counted at 40X magnification (fig. 2.1). Immotile sperm were not counted. The percentage of bound sperm is the hyaluronan binding capacity

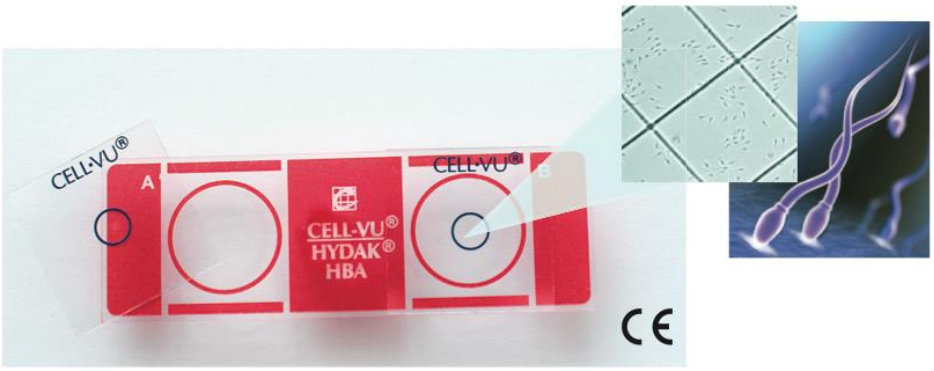


Figure 2.8 Sperm Hyaluronan Binding Assay (HBA)

Sperm exposed to glycation conditions were place on HBA slides and assessed for HA binding. Bound sperm are differentiated from unbound sperm by their beating tails with heads that make no progressive movement (*from Origio.com*).

of the cells. Sperm motility was also assessed at these stages using a computer assisted sperm analyser (CASA) recording grades A (fast progressive motility), B (slow progressive motility), C (non-progressive motility) and D (immotile).

2.5.11 CML levels in obese subjects

To determine whether obese individuals have higher levels of sperm AGEs, CML was measured in obese men and compared with that of normal weight individuals. 15 men were recruited via local newspaper advertisements and word of mouth. Nine of the participants were obese according to World Health Organization guidelines (WHO, 2013), having a BMI ≥ 30 kg/m^2, and six participants had a normal BMI of 18-24 kg/m^2. The obese and normal-BMI groups were not age-matched due to the low number of volunteers. Semen analysis was carried out as described in section 2.5.1. Three of the subjects had sperm count of <10x10^6 cells/ml and did not have the minimum required for the CML assay. One individual could not produce a sample, resulting in five participants for each group. The immunoassay was run as detailed in section 2.5.6 and stained cells were analysed by flow cytometry.

2.6 *In vitro* glycation of hyaluronidase protein

To determine the effects of glycation on the SPAM1 hyaluronidase activity, firstly molecular cloning was carried out to generate the recombinant form of the protein. However, this was not successful, and so a commercially available hyaluronidase, Cumulase™ (Origio, Denmark), was used. This enzyme is used in preparation for ICSI to disperse the cumulus cells in retrieved oocytes. Furthermore, to determine the effects of glycation, a hyaluronidase substrate gel assay was developed to measure enzyme activity.

2.6.1 Generation of recombinant SPAM1 protein

2.6.1.1 Molecular cloning

A pENTR223 vector containing cDNA insert of sperm adhesion molecule 1 (SPAM1/PH-20/hyaluronidase) was obtained from the Harvard Medical School PlasmID Repository (Clone ID: HsCD00375886). The SPAM1 insert was amplified by PCR using primers designed based on the full length SPAM1, transcript variant 1 (XM_011516523). SPAM1F: 5'-TGGCATGGATCCATGGGAGTGCTAAAATTCAAGCAC -3', and SPAM1R: 5'- TGGCAAGTCGACGAAGAAACCAATTCTGCTAATA -3'. Briefly, 1 ng plasmid DNA was added to SPAM1 primers (0.3 µM) and 5x MyTaq Reaction Buffer (Bioline, UK). Cycling conditions included an initial denaturation at 95°C for 15 minutes, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 61°C for 30 s, extension at 72°C for 30 s, and a final extension of 72°C for 5 minutes.

The PCR products were separated on a 0.8% agarose gel at 50 volts for 1 hour and visualised under ultraviolet (UV) light using a Gel Imaging System G-BOX (BioRad). The product was expected to be 1533 bp. Bands were excised and cleaned up using QIAquick Gel Extraction Kit (Qiagen, UK). Molecular cloning was carried out at the Laboratoire de Biologie du Développement de Villefranche-sur-mer, Sorbonne Universités. Briefly, sequences were cloned into the pGEX 4T1 vector, which contained a glutathione-S-transferase (GST) tag sequence. A restriction digest was performed on pGEX 4T1 vector using the restriction enzymes BamHI and SalI and the SPAM1 insert was ligated into the multiple cloning site. Clones were transformed into *E.coli* BL21 RiPL cells (Agilent, UK) using heat shock for 2 minutes at 42°C followed by plunging on ice. Cells were plated out onto liquid broth (LB) agar (10% tryptone, 5% yeast extract, 10% NaCl) with ampicillin (1 mg/ml) (Thermo Scientific, UK) and allowed to grow overnight at 37°C.

2.6.1.2 Bacterial cell culture and induction with IPTG

A single successful recombinant colony was inoculated into 5 ml LB media (10% tryptone, 5% yeast extract, 10% NaCl) and allowed to grow overnight at 37°C, with shaking for aeration at 270 rpm (Thermo Scientific, UK) . The following day, 0.5 ml or 5 ml overnight culture was added to 50 ml LB media or 1 L LB media and grown for 2.5 hours until the log growth phase. When an OD600 value between 0.4 and 0.6 nm was reached, 1 ml of non-induced culture was removed and saved for later analysis while the remaining cells were induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, UK). Cells were harvested at 2 and 5 hours post-induction. Later, to reduce the formation of inclusion bodies, cells were grown as above and allowed to cool to room temperature before induction with IPTG, followed by growth at 16°C or 20°C overnight with shaking at 270 rpm. Cells were centrifuged at 600 x g for 10 minutes and pellets were frozen until lysis.

2.6.1.3 Protein purification

Bacterial cells were lysed in 1xPBS containing lysozyme (1 mg/ml) and Triton X-100 (0.2%) on ice for 30 minutes. DNase and RNase were added to a final concentration of 5 µg/ml to reduce the viscosity of the solution. Cells were spun down to separate soluble protein, and the insoluble pellet was resuspended in a detergent-based lysis buffer containing N-lauroylsarcosine (1.5%), diethanolamine (25 mM) and EDTA (1 mM). The GST-SPAM1 recombinant proteins were purified on a column using Glutathione Sepharose 4B (Sigma, UK) and eluted with 20 mM reduced glutathione (50 mM Tris, pH 8.0) (Sigma, UK). Protein could not be successfully purified so the experiment was terminated here and a commercial hyaluronidase enzyme was used.

2.6.2 Hyaluronidase substrate gel assay

2.6.2.1 Assay development

A substrate gel assay was developed to quantify hyaluronidase activity of the commercial enzyme Cumulase™ (Origio, UK) by polyacrylamide gel electrophoresis (PAGE). A 10% polyacrylamide separating gel was prepared using 30% acrylamide/bisacrylamide. Hyaluronic acid (10 mg/ml) was dissolved in water at 4°C for 24 hours and added to the separating gel mix to a final concentration of 0.1 mg/ml. A 7.5% stacking gel was prepared without hyaluronic acid. Gels were loaded with 5-10 µg of Cumulase protein™ and run at 15 mA per gel for 1 hour 40 minutes. Following electrophoresis, gels were washed in 3% Triton X-100 in 50 mM HEPES (pH 7.4) for 2 hours on a benchtop rotator to remove SDS. A number of buffers were tested for enzymatic activation of the hyaluronidases: 0.1 M sodium formate (pH 3.75) with 0.15 M NaCl; 50 mM HEPES (pH 7.4) with 0.15 M NaCl; 12.5 mM sodium acetate (pH 3.0), 25 mM sodium acetate (pH 4.0), 50 mM sodium acetate (pH 6.0) and 100 mM sodium acetate (pH 7.0). Gels were incubated with the buffers for 16-39 hours, followed by staining with Alcian blue (0.5%, 3% acetic acid) for 2 hours. Gels were destained in 7% acetic acid until clear bands could be seen on the gels. Following this, protein bands were stained using Coomassie blue (0.5%, 50% methanol, 10% acetic acid) and destained in 50% methanol, 10% acetic acid. Gels were visualised and photographed on a Chemidoc touch transilluminator (Bio-Rad, UK). Band sizes were analysed using Image Lab (Bioline, UK).

2.6.2.2 Glycation of Cumulase™

Cumulase™ protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, UK) using bovine serum albumin (BSA) standards provided with the kit. Glycating agents MG (5 µM), GO (5 µM), glucose (20 mM), fructose (20 mM) and mannitol (20 mM) were then diluted in PBS (pH 7.4) (Fisher, UK) with sodium azide (0.02 mM) and added

to an equal volume of Cumulase™ enzyme (0.5 mg/ml). A sample of Cumulase™ enzyme with PBS alone was included (0.5 mg/ml) to account for non-glycation induced changes in the protein. A sample of BSA protein alone with PBS was also included to account for support protein activity as there is human serum albumin (HSA) present in Cumulase™. Samples were incubated at 37°C and 50 µl aliquots were removed at days 0, 3, 7 and 14 and snap frozen in liquid nitrogen until measurement.

2.6.2.3 Measurement of glycated Cumµase™ using the hyaluronidase substrate gel assay

A 10% polyacrylamide separating gel containing HA and 7.5% polyacrylamide stacking gel were prepared as described in section 2.6.2.1. Cumulase samples were mixed with sample buffer at a 4:1 ratio and 20 µl of this was loaded on to the gels. Gels were run at 15 mA per gel for 1 hour 30 minutes. Gels were treated for hyaluronidase activity as described in section 2.6.2.1, with the exception that the hyaluronidase assay buffer used was 25 mM sodium acetate (pH 4.0) for all gels, and this was carried out at 37°C for 16 hours as described.

Chapter 3

Effects of lifestyle and environment on DNA methylation and infertility

3.1 Introduction

Over half of male factor infertility cases are idiopathic and the discovery of differences in methylation between fertile and infertile men has led to speculation that this could be one of the causes (Hammoud *et al.,* 2010; Aston *et al.,* 2015). DNA methylation is a major regulatory mechanism for gene expression and primarily silences genes by preventing transcription factor interaction with DNA at important regulatory sites, such as promoters. Several lifestyle factors have been linked to changes in the sperm DNA methylation as well as to aberrant semen parameters, including age, obesity, alcohol consumption, nutrition, physical activity and exposure to environmental toxicants (Jenkins *et al.,* 2014; Donkin *et al.,* 2015; Ouko *et al.,* 2009; Aarabi *et al.,* 2015; Denham *et al.,* 2015; Chiu *et al.,* 2015; De Rosa *et al.,* 2003). Therefore, current research seeks to determine whether the decrease seen in male fertility is linked to lifestyle-induced changes in the methylome.

Much of the research on sperm methylation has focussed on imprinted genes (Kobayashi *et al.,* 2007; Boissonnas *et al.,* 2010; Geuns *et al.,* 2007; Ankolkar *et al.,* 2013; Poplinski *et al.,* 2009). Imprinting is the process of silencing or activating certain genes in a parent of origin-specific manner, to confer mono-allelic gene expression. These genes are crucial to ensure the correct balance of gene expression in the developing embryo, which if disturbed results in developmental abnormalities (Jacob *et al.,* 2013) and DNA methylation is the main regulatory mechanism controlling this (Kaneda *et al.,* 2004). As many studies have reported differences in imprinted gene

methylation between the sperm of fertile and infertile men, a major question resides over whether this has arisen due to abnormal reprogramming during germ cell development, which could be a source of deregulated gene expression leading to infertility, or alternatively, whether sperm epimutations are acquired during post-natal life from lifestyle and environmental exposures, leading to aberrant methylation.

DNA methylation also varies at non-imprinted genes important for regulating epigenetic machinery or important in sperm function. The 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene codes for a key enzyme involved in folate metabolism, the pathway by which methyl groups are obtained for DNA methylation. Hypermethylation of the *MTFHR* promoter has been found in infertile men (Wu *et al.,* 2010; Botezatu *et al.,* 2014), and critically, this hypermethylation has been linked to hypomethylation at other genes in infertile men (Rotondo *et al.,* 2013) suggesting a disruption of the methylation process itself. Furthermore, array-based sequencing studies have shown that fertile and infertile men actually have very distinct methylation patterns across numerous genes with varying roles (Houshdaran *et al.,* 2007; Urdiguino *et al.,* 2015; Shütte *et al.,* 2013). These differences in normal DNA methylation may have implications for offspring health and may be a mechanism behind the transgenerational effects of male health (Northstone *et al.,* 2014; Axelsson *et al.,* 2013; Soubry *et al.,* 2015). Understanding the transmission of non-genetic factors through the male germ line is critical to elucidating the origins of disease.

The “deleted in azoospermia-like” (*DAZL*) gene encodes an RNA-binding protein which is essential for gametogenesis. Disruption of *Dazl* in *C. elegans*, causes meiotic arrest in oogenesis (Karashima *et al.,* 2000), in Xenopus, is required for early primordial germ cell differentiation (Houston and King, 2000), and in mice, leads to spermatogenic arrest (Schrans-Stassen *et al.,* 2001). In humans a lack of *DAZL* transcript causes azoospermia (Lin *et al.,* 2001). Importantly *DAZL* expression is regulated by DNA methylation at a CpG island at its promoter, which is unmethylated in reproductive cells (Linher *et al.,* 2009). A few recent studies have shown that

alterations in *DAZL* DNA methylation closely associate with spermatogenesis disorders in patients with infertility (Navvaro-Costa *et al.,* 2010), suggesting this may be a strong candidate gene for spermatogenic failure.

Assisted reproductive techniques (ART) bypass the selection processes that happen during normal fertilisation, particularly in ICSI when a single sperm is injected into the oocyte which circumvents sperm binding and penetration of the ZP. Therefore, there is much interest in whether aberrant DNA methylation signatures in the sperm of infertile men could have consequences and for fertilisation rates and for success of the developing embryo. As there is some evidence of an increase in imprinting disorders following ART, it is possible that aberrant sperm methylation could play a role (Lazaraviciute *et al.,* 2014). Indeed, some studies have shown that methylation errors in sperm are associated with poor ART outcomes (Kuhtz *et al.,* 2014) and even that the same epimutations have been found in the offspring as in the paternal sperm DNA (Kobayashi *et al.,* 2009). However, other research shows that aberrant embryonic methylation is not linked to sperm DNA methylation (Camprubi *et al.,* 2012) and aberrant sperm methylation does not affect ART outcomes (Ibala-Romdhane *et al.,* 2011). As more than 5 million babies have been born through ART, this is an important area of public health research that requires further study.

3.1.1 Aims

The aims of this chapter were to:

1. Identify the associations between sperm DNA methylation, health, lifestyle, and chemical or physical hazard exposure.
2. Determine whether sperm DNA methylation is associated with aberrant semen parameters.
3. Determine whether differences in sperm DNA methylation are associated with ART outcomes.

3.2 Results

3.2.1 DNA methylation and lifestyle

3.2.1.1 Global and gene-specific sperm DNA methylation

Percentage DNA methylation is binary, i.e. methylated or not and refers to the proportion of DNA copies (i.e. cells) that are methylated. Firstly, the descriptive statistics were analysed for each gene. Table 3.1 shows the mean methylation of all neat semen samples. The paternally imprinted genes *H19* exon 1 and *MEG3* were hypermethylated, showing close to 100% methylation. *H19* CTCF6 showed a mean methylation of 40.06% with a large range including hypomethylated (6.2%) and hypermethylated (76.2%) samples. The maternally imprinted genes *PLAGL1* and *SNRPN* were hypomethylated, having methylation levels close to 0%. The non-imprinted genes *MTHFR* and *NR3C1* also had low levels of methylation, and the spermatogenesis-associated gene *DAZL* had a low mean methylation with a large amount of variation ranging from 0.42% to 20%.

Table 3.1 DNA methylation summary table

Raw data for global and gene specific DNA methylation (%) is shown. Patients with varicocele (n=3) were excluded.

DNA methylation	Mean(SD)	Median	25th percentile	75th percentile	Min	Max	n
5MedCyd (nM)	381.58(95.75)	375.29	328.45	432.16	163.24	719.54	66
H19 CTCF6 (%)	40.07(13.82)	39.95	29.8	47.55	6.2	76.2	92
H19 exon 1 (%)	90.85(2.94)	91.5	90.8	92.05	76.0	93.8	69
PLAGL1 (%)	3.31(0.39)	3.29	3.11	3.54	2.4	4.14	72
SNRPN (%)	2.79(2.01)	2.17	1.67	2.87	1	12.33	86
MEG3 (%)	88.12(2.12)	88.5	87.2	88.5	81.5	91.7	87
MTHFR (%)	1.76(0.42)	1.67	1.55	2.0	1	3	80
DAZL (%)	3.92(3.91)	2.58	1.83	3.83	0.42	20	71
NR3C1 (%)	5.14(0.97)	5.08	4.57	5.86	3.29	8.43	74

3.2.1.2 CpG site-specific methylation

In many genes, certain CpG sites in a regulatory region can be more important than others. Individual CpG site methylation was analysed within each gene region using box and whisker plots to identify sites with greater variability, and therefore more likelihood of demonstrating lifestyle-influenced changes. CpG site methylation was also correlated with mean methylation across all sites in the region and Pearson's correlation (r) values were analysed to identify those sites that differed from the mean trend (table 3.2). In sperm, at paternally imprinted genes, patients that show low levels of methylation are considered aberrant, while at maternally imprinted genes, those that show higher levels of methylation are considered aberrant.

H19 CTCF6

H19 is a paternally imprinted, and therefore methylated, gene. The 6th CTCF binding site exhibits variable methylation due to its role in CTCF binding. Figure 3.1 A shows the box and whisker diagrams for the 6th CTCF binding site (CTCF6). There are 30 CpG sites overall in the 6th CTCF binding site and these sites represent CpGs 5-12 within that region. There was a large amount of variation across all sites with the largest SDs at CpGs 4, 6 and 8 and the smallest SD at CpG 3, which also had the lowest mean methylation at 25.51% (table 3.2). The mean methylation of all the CpG sites was 40.07 (table 3.1) and all individual CpG sites were highly correlated with this (p<0.0001). The extreme cases in the 95th percentile and 5th percentile all appeared at more than 1 CpG site and no cases present in the 95th percentile appeared in the 5th percentile at any sites, and vice-versa. Therefore, generally samples were hyper- or hypo-methylated at several CpG sites. All cases that were in the 5th percentile had normal semen parameters.

H19 exon 1

H19 exon 1 showed methylation levels more typical of a paternally imprinted gene, i.e. heavily methylated (fig. 3.1 B). All CpG sites in this region showed

hypermethylation with CpG sites 1, 2, 4 and 5 having mean methylation above 90% and CpG 3 having the lowest methylation at 86.45% and the largest SD at 4.43 (table 3.2) which signifies the largest and most deviant amount of variation in the population, however this site along with all others was highly correlated with the mean CpG methylation (p<0.0001). Cases that appeared most frequently in the 5th percentile (low methylation) were more likely to be oligozoospermic than those in the 95th percentile: three out of five patients that appeared in the 5th percentile were oligozoospermic. Some of the cases in the 95th percentile of exon 1 were also in the 95th percentile of the 6th CTCF binding site, though not all. None of the cases in the 5th percentile were present in the 5th percentile of the 6th CTCF binding site. It seems likely, therefore that these two sites are operating differently.

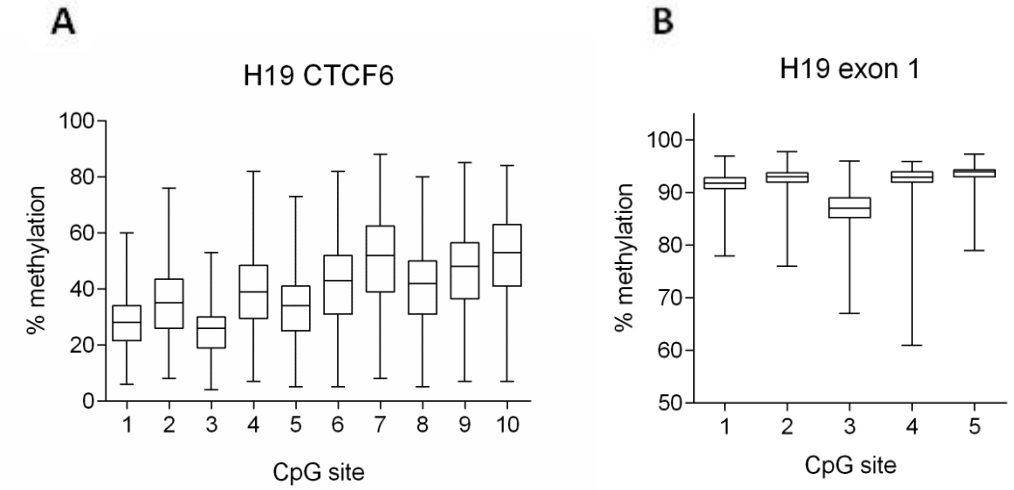


Figure 3.1 *H19* CTCF6 and exon 1 box and whisker plots

CpG site-specific methylation (%) is shown for the *H19* gene at the 6th CTCF binding site (A) and at exon 1 (B). Boxes show the median, 25th and 75th percentiles.

PLAGL1

PLAGL1 and *SNRPN* are maternally imprinted genes, therefore in sperm they exhibit low levels of methylation. Figure 3.2 A supports this as *PLAGL1* showed mean methylation less than 5% at all CpG sites. All sites showed

similar SDs (0.5-0.78%) and all were highly correlated with the mean CpG methylation (p<0.0001).

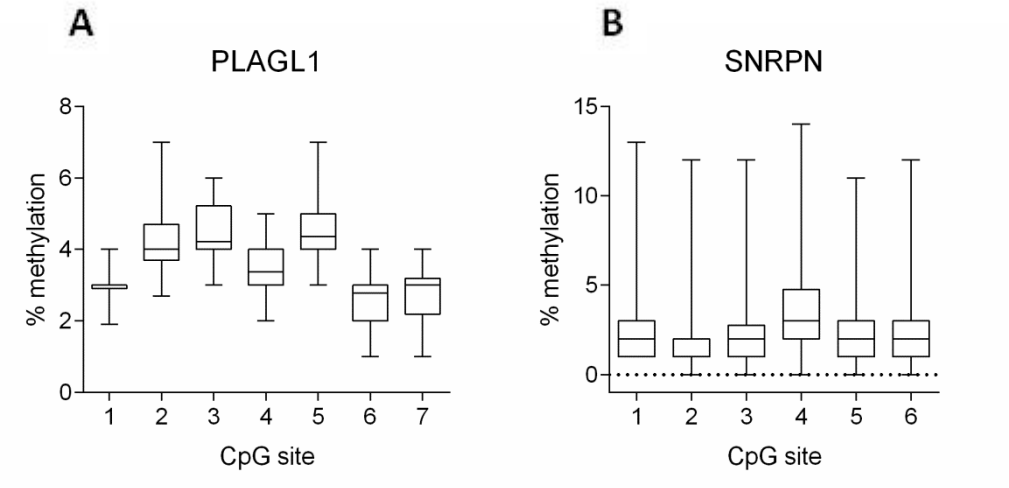


Figure 3.2 *PLAGL1* and *SNRPN* box and whisker plots

% DNA methylation at individual CpG sites is shown for the maternally imprinted *PLAGL1* (A) and *SNPRN* (B) genes. Boxes show the median, 25th and 75th percentiles.

SNRPN

SNRPN methylation showed a large amount of variation at all CpG sites, with many patients showing higher than average methylation which can be seen by the extension of the upper whisker (fig. 3.2 B). In the 95th percentile, two out of four patients were oligozoospermic, and in the 5th percentile, one out of two patients was oligozoospermic. The cases in the 95th percentile were not the same cases as those for *PLAGL1* suggesting not all individuals showed hypermethylation across all sites. Furthermore, patients in the 5th percentile were not found in the 95th percentile in the paternally imprinted genes *H19* and *MEG3*, which suggests that aberrant de-methylation at paternal imprints is not related to aberrant re-methylation at maternal imprints. All CpG sites were highly correlated with the mean (p<0.0001).

MEG3-IG DMR

Like H19, *MEG3* is also a paternally imprinted gene, and shows close to 100% methylation (fig. 3.3). CpGs 6, 7 and 9 had the lowest methylation means at 69.49% (±3.83), 69.02% (±7.46), 54.93% (±5.34). CpG 8 had the largest SD at 10.77%. All sites were highly significantly associated with the mean CpG methylation ($p<0.0001$) except for CpG 6 ($p<0.01$) and CpG 9 which was not correlated with the mean ($p>0.05$). Therefore the two sites of interest would be CpG 8 which has the largest variation and CpG 9 which deviates largely from the mean. Two patients which were hypomethylated at CpG 7 (12%) and CpG 9 (16%) were normozoospermic. The individuals in the 5th percentile for *MEG3* were not consistently in the 5th percentile at all CpG sites, unlike for *H19*. Two of the cases that were in the 5th percentile for *MEG3* were also in the 5th percentile of *H19* exon 1, however none matched the cases in the 5th percentile of *H19* CTCF6.

MTHFR promoter

MTHFR is a non-imprinted gene whose transcript is critical to the methylation cycle itself, and its promoter therefore shows low levels of methylation (fig. 3.3 B). The CpG sites showed methylation levels of 1.93% (±0.68), 1.24% (±0.48) and 2.5% (±0.71). The variation (SD) was similar across the sites and all sites correlated significantly with the mean ($p>0.0001$). As higher *MTHFR* methylation could lead to lower methylation at other genes, similarities between patients in the 95th percentile of *MTHFR* were compared with those in the 5th percentile of other genes but no associations were found.

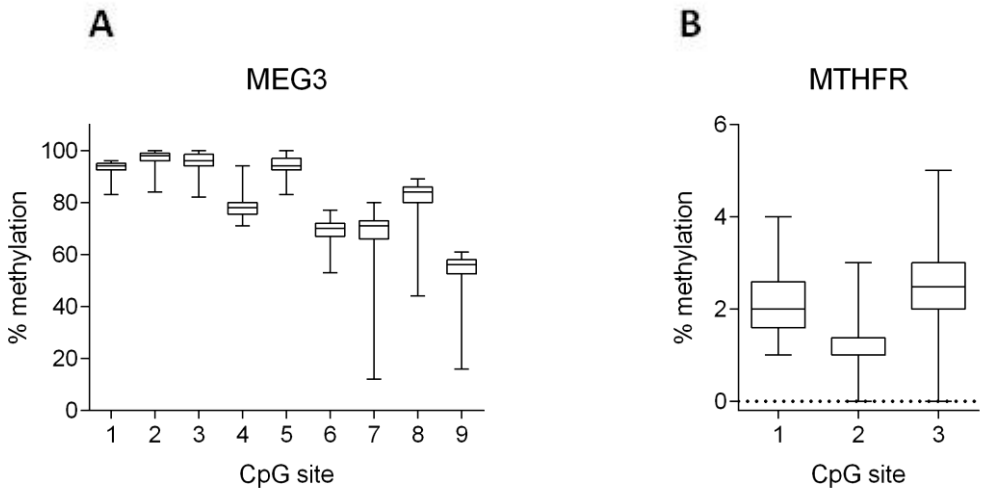


Figure 3.3 *MEG3 and MTHFR* box and whisker plots

% DNA methylation at individual CpG sites is shown for the paternally imprinted *MEG3* gene (A) and *MTHFR* gene (B). Boxes show the median, 25th and 75th percentiles.

NR3C1

The *NR3C1* gene is involved in the stress pathway and is not an imprinted gene. The promoter usually shows about 5% methylation in sperm. CpG sites 1-4 showed large levels of variation which is clear from the SDs (fig 3.4 A and table 3.2). CpG 2 had the highest mean methylation (8.9%) and the largest SD (2.64%). CpGs 5-7 had low levels of methylation and very small SDs. CpG 5 had the lowest methylation 1.36% and the smallest SD (0.81%). CpGs 5-7 may be important CpG sites as their variation is low, therefore tightly controlled, while CpGs 1-4 might be most affected by lifestyle and environmental stimuli. There was no consistency in patients appearing in the 95th or 5th percentiles indicating no blanket hypo- or hyper-methylation for all sites.

DAZL

The *DAZL* gene has important roles in spermatogenesis and therefore normally has low levels of methylation in sperm. *DAZL* showed large levels of variation across all CpG sites. Mean methylation was below 7% at all CpGs however the upper range was extremely large indicating many men with hypermethylation of this gene (fig. 3.4 B). Participants with the highest levels of methylation were consistently heavily methylated at all CpG sites. In the 95th percentile, three out of four men were oligozoospermia, while none of the participants in the 5th percentile were. There was no association between hypermethylated sites in *DAZL* and hypomethylation at paternally imprinted genes or hypermethylation at maternally imprinted genes.

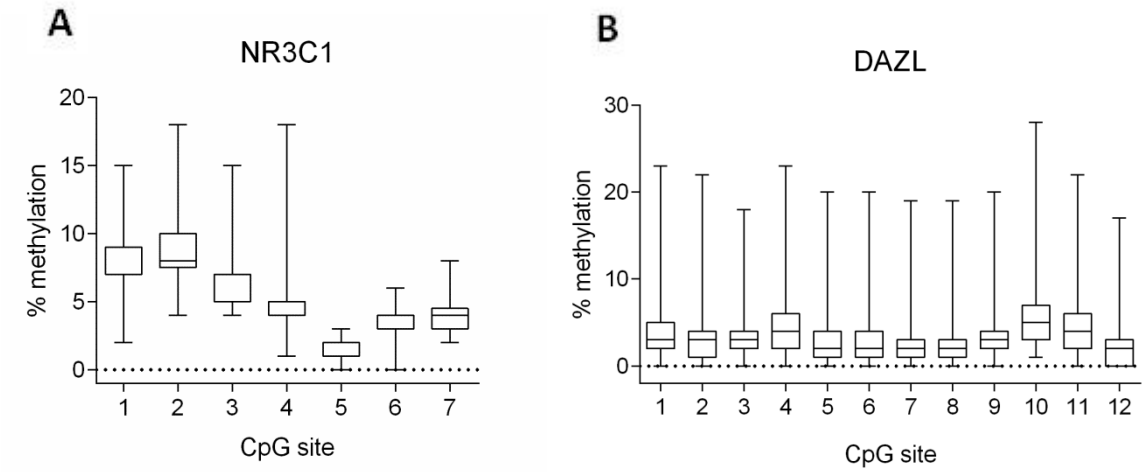


Figure 3.4 *NR3C1* and *DAZL* box and whisker plots

% DNA methylation at individual CpG sites is shown for the non-imprinted genes *NR3C1* (A) and *DAZL* (B). Boxes show the median, 25th and 75th percentiles.

	1	2	3	4	5	6	7	8	9	10	11	12
H19 CTCF6 (n=93)												
Mean	29.01	35.8	25.51	40.88	34.44	51.69	41.27	47.35	52.13	40.09	--	--
SD	11.06	13.14	9.35	15.46	13.5	15.72	15.06	15.38	15.62	13.75		
r	0.95****	0.96****	0.95****	0.99****	0.99****	0.99****	0.98****	0.99****	0.99****	0.98****		
H19 exon 1 (n=69)												
Mean	91.34	92.67	86.45	92.26	93.49	--	--	--	--	--	--	--
SD	2.3	2.58	4.43	4.35	2.17							
r	0.77****	0.87****	0.74****	0.66****	0.74****							
MEG3 (n=87)												
Mean	92.98	97.22	96.11	77.97	94.76	69.49	69.02	80.29	54.93	--	--	--
SD	2.84	2.32	3.37	3.67	3.52	3.83	7.46	10.77	5.34			
r	0.69****	0.49****	0.62****	0.52****	0.61****	0.26**	0.7****	0.72****	0.03			
SNRPN (n=88)												
Mean	2.68	2.49	2.31	3.91	2.53	2.63	--	--	--	--	--	--
SD	2.3	2.33	2.14	2.8	1.84	2.05						
r	0.9****	0.9****	0.91****	0.91****	0.82****	0.9****						
PLAGL1 (n=83)												
Mean	2.86	4.02	4.67	3.37	4.54	2.65	2.79	--	--	--	--	--
SD	0.53	0.78	0.71	0.61	0.7	0.68	0.61					
r	0.73****	0.73****	0.65****	0.76****	0.67****	0.85****	0.73****					
MTHFR (n=83)												
Mean	1.93	1.24	2.5	--	--	--	--	--	--	--	--	--
SD	0.68	0.48	0.71									
r	0.59****	0.5****	0.8****									
DAZL (n=71)												
Mean	4.44	4.07	3.85	4.89	3.62	3.24	2.97	3.06	3.54	6.3	4.11	1.85
SD	4.67	4.42	3.79	4.79	4.04	3.6	3.63	3.54	3.51	4.92	6.26	3.45
r	0.9****	0.97****	0.9****	0.93****	0.96****	0.95****	0.97****	0.96****	0.95****	0.92****	0.92****	0.97****
NR3C1 (n=73)												
Mean	7.81	8.9	6.16	4.63	1.36	3.95	5.13	--	--	--	--	--
SD	2.15	2.64	2.3	2.11	0.81	1.18	0.97					
r	0.58****	0.67****	0.59****	0.56****	0.3*	0.38***	0.49****					

Table 3.2 Individual CpG site methylation (%)

Means and SD were calculated for all participants at each CpG site. Correlations (r) were made between CpG site methylation and mean methylation across all CpG sites in each gene to identify those which did not follow the mean changes. Significant correlations are shown in bold. *p<0.05

3.2.1.3 Normalisation of DNA methylation data

In order for parametric statistical analysis to be carried out, data must show a Gaussian, or normal, distribution, typified as a bell-shaped curve on a frequency distribution graph. Many of the genes showed positively or negatively skewed data which require transformations to a Gaussian distribution.

For example, *SNRPN* was highly positively skewed and a reciprocal transformation $(1/x)$ was carried out (fig. 3.5 A and B). *DAZL* also underwent a reciprocal transformation. *NR3C1* was $\log^{10}$ transformed and *MEG3* was reversed and $\log^{10}$ transformed. 5MedCyd, *H19* CTCF6. *H19* exon 1, *MTHFR* and *PLAGL1* did not require transformation.

Table 3.3 shows the means and 95% confidence intervals for global and gene-specific methylation following transformation. Geometric means were calculated either from original methylation values if the data was not transformed, or by back-transforming the means and 95% CI of data that was transformed.

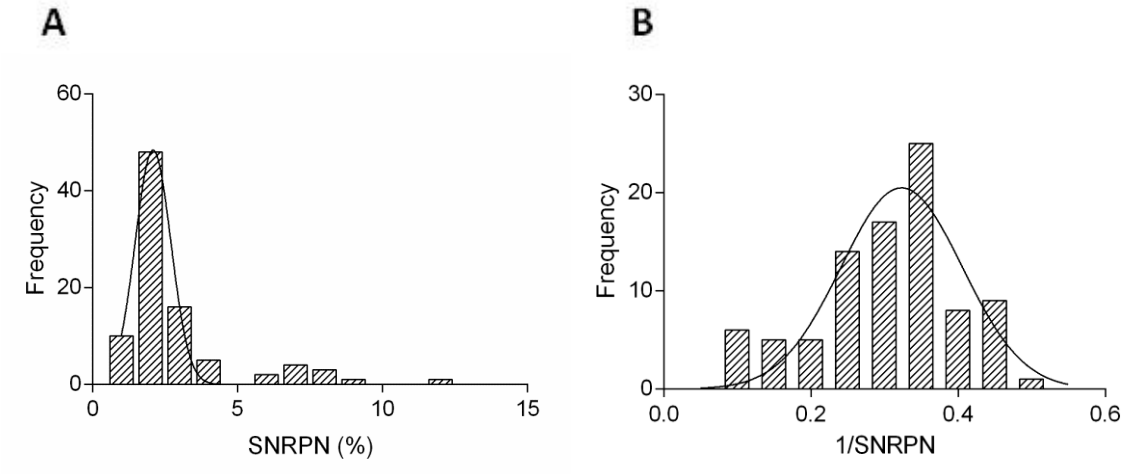


Figure 3.5 Normalisation of *SNPRN* data

Histograms illustrating the normalisation of SNRPN data from a highly positively skewed distribution (A) to a more Gaussian distribution (B) (n=85).

Table 3.3 Summary of transformed global and gene-specific methylation data

DNA methylation	Transformation	Geometric Mean	95 % CI		n
			Lower	Upper	
5MedCyd (nM)	None	381.58	358.04	405.12	66
H19 CTCF6 (%)	None	40.07	37.2	42.93	92
H19 exon 1 (%)	None	91.24	90.66	91.82	66
PLAGL1 (%)	$Log^{10}((MEG3_I+1)-MEG3_x)$	88.57	88.14	88.96	87
SNRPN (%)	1/(SNRPN+1)	2.26	2.06	2.49	85
MEG3 (%)	None	3.54	3.43	3.65	79
MTHFR (%)	None	1.89	1.8	1.98	79
DAZL (%)	1/(DAZL+1)	2.24	2.4	3.12	69
NR3C1 (%)	Log^{10}(hNR3C1+1)	5.06	4.85	5.28	74

3.2.1.4 Participant demographics, health and lifestyle

Table 3.4 Frequencies of exposures 24 hours prior to sample collection

Variable	Number (%)
Smoke exposure in last 24 h	
Not exposed	86 (89.6)
Exposed	10 (10.4)
Work attendance	
No attendance	28 (28.9)
Attendance	69 (71.1)
Red meat consumption 24 h	
No	47 (48.5)
Yes	50 (51.5)
White meat consumption 24 h	
No	59 (60.8)
Yes	38 (39.2)
Fish consumption 24 h	
No	72 (74.2)
Yes	25 (25.8)

Participants completed a questionnaire regarding their health, lifestyle and exposures. In the first section of the questionnaire (appendix 1), participants were asked about their exposure to secondary cigarette smoke, attendance at work and diet in the 24 hours prior to giving their sperm sample (table 3.4). In the second part of the questionnaire, participants were asked about their health and lifestyle (tables 3.5 and 3.6) and exposure the certain chemical and physical agents (table 3.7). An initial exploration of the questionnaire results was carried out to examine trends in the data set.

Table 3.5 Participants health and lifestyle 3 months prior to the study

Variable	Number (%)
Fever or flu	
No	84 (86.6)
Yes	13 (13.4)
Job illness	
No	81 (83.5)
Yes	16 (16.5)
Illness due to stress	
No	88 (90.7)
Yes	9 (9.3)
Smoking history	
Non-smoker	50 (52.6)
Ex-smoker	45 (47.4)
Alcohol consumption	
>1 drink per week	30 (30.9)
<1 drink per week	67 (69.1)
Underwear	
Boxer shorts not worn	7 (7.4)
Boxer shorts worn	88 (92.6)
Leisure score index	
<24 points	52 (54.2)
≥24 points	44 (45.8)
BMI (kg/m^2)	
<25	46 (51.7)
25-29.9	29 (32.6)
≥30	14 (15.7)

The median age was 36 years (25 to 57 years), however most ages clustered closer to the median as the 25th and 75th percentiles were 32 and 39, respectively (table 5). The study population was mostly White men with one Black “other” male and one Pakistani male. 10.4% of men were exposed to smoke in the previous 24 hours, and 71.1% had attended work. Red meat was the most common type of meat consumed in the previous 24 hours (51.5%) compared with white meat (39.2%) and fish (25.8%).

Table 3.6 Continuous participant demographics

Variable	Mean (SD)	n	Median	25th percentile	75th percentile	Min	Max
Age (years)	36.05 (5.4)	96	36.0	32	39	25	57
BMI (kg/m^2)	25.73 (3.73)	93	25.18	22.95	27.61	17.51	37.22
Alcohol (units/week)	9.94 (10.2)	97	9.2	0	15.6	0	52
Leisure score index	24.15 (23.3)	96	23.0	5	37.75	0	124
Smoking (years)	12.9 (7.13)	45	11.0	7.5	18.5	0.5	33

Thirteen participants (13.4%) reported to have had fever or flu in the 3 months prior to the study, and 6/13 of these reported cases also had an illness that was caused by or made worse by their job (table 3.5). Sixteen individuals reported to have a work-related illness and half of these were due to stress, depression or anxiety (n=8) and sleep problems (n=3). Other reasoning included breathing or lung problems (n=3), headaches and/or eyestrain (n=5), bone, joint or muscle problems affecting arms/hands/neck/shoulders (n=3), hips, legs or feet (n=1) or back (n=1), however these were not excluded from further analyses as these physical problems are not relevant to sperm DNA methylation. The smoking history of patients consisted of non-smokers (52.6%) or ex-smokers (47.4%) and the number of years of smoking was considered due to the consequences of long term smoking on health (table 3.6). The median BMI was 25.18 kg/m^2 with around 51.7% of men having a normal weight, 32.7% being overweight

and 15.7% being obese; furthermore, roughly 50% of men were physically active and 50% were insufficiently active (table 3.5). Alcohol consumption was moderate as although 69.1% of men reported to consume more than one alcoholic drink per week (table 3.5), the number of units consumed were relatively low: the 75th percentile was 15.6 units (table 3.6) which is considerably lower than the weekly recommended limit of 21 units.

Table 3.7 Participants exposure to chemical or physical agents at work or at home 3 months prior to the study

Exposure	Number (no/yes)	% yes
Metal dust or fumes	75/20	21.1
Pesticides	93/2	2.1
Herbicides	87/8	8.4
Fertilisers	86/8	8.5
Oils or greases	69/26	27.4
Detergents and soaps (at job only)	59/36	37.9
Glues, adhesives or resins	67/28	29.5
Paints, varnishes or lacquers	69/26	27.4
Printing inks or dyestuffs	85/10	10.5
Dry cleaning fluids	94/1	1.1
PVC or plasticisers (at job only)	91/4	4.2
Other solvents	71/24	25.3
Extremely hot environments	87/8	8.4
Heavy vibrating machinery	78/17	17.9
Nonionizing radiation	90/5	5.3
Driving for long periods	82/4	4.7
X-rays	83/3	3.5

Frequencies and percentages of exposure to each chemical or physical agent are shown. Heavy vibrating machinery includes equipment or vehicles.

Participants were questioned about their exposure to various chemical and physical hazards (table 3.7). Some participants additionally reported long periods of driving and exposure to X-rays and these are included in the summary data. Exposure to pesticides, herbicides, fertilisers, dry cleaning fluids, PVC/plasticisers, hot environments, non-ionizing radiation, driving and X-rays was low, with less than 10% of men exposed. These variables were not included in any further analysis due to the low statistical power and false positives that low numbers could generate. The most frequent exposures were metal dust or fumes, oils or greases, detergents and soaps, glues and adhesives and resins, paints and varnishes and lacquers, printing inks or dyestuffs, solvents, and heavy vibrating machinery, equipment or vehicles.

3.2.1.5 DNA methylation and lifestyle factors

3.2.1.5.1 Sperm DNA methylation and age, BMI, smoking duration (years) and alcohol use

Current literature suggests that with increasing age, global DNA methylation decreases, while gene-specific methylation may become hypo- or hyper-methylated. In this study, spearman's correlation revealed that neither global nor gene-specific methylation was correlated with age (table 3.8). When only men classed as fertile upon consultation at the hospital were investigated, global methylation still did not show a significant change with age (Spearman's $\rho = -0.329$; $p=0.146$; n=21). When infertile men were investigated, global methylation was not significantly different with age (Spearman's $\rho=-0.137$; $p=0.387$; n=42).

The associations between BMI, duration of previous smoking, physical activity levels (LSI) and weekly alcohol consumption with DNA methylation were also investigated. Spearman's correlation revealed no significant correlations between these variables and DNA methylation (table 3.9).

Table 3.8 Age and sperm DNA methylation

Gene or region	ρ-coefficient	Sig. (p-value)	n
5MedCyd	-0.111	0.374	66
H19/CTCF6	-0.027	0.797	90
H19 exon 1	-0.167	0.17	69
PLAGL1	0.054	0.639	78
MEG3	-0.016	0.883	87
SNRPN	0.141	0.188	89
hNR3C1	-0.021	0.861	74
MTHFR	0.015	0.895	79
DAZL	-0.003	0.983	70

Spearman's correlation, ρ, was carried out for global methylation and for each gene or region with age. Age was not related to DNA methylation.

Table 3.9 DNA methylation and lifestyle correlations

	Spearman's correlation, ρ (*p*-value)			
	BMI	LSI	Smoking (years)	Alcohol (units/wk)
5MedCyd	0.017(0.897)	0.071(0.583)	-0.01(0.938)	-0.025(0.849)
n	59	62	62	62
H19 CTCF6	-0.188(0.081)	-0.017(0.876)	-0.135(0.214)	0.04(0.708)
n	87	89	86	91
H19 exon 1	0.044(0.722)	0.023(0.85)	-0.104(0.395	-0.185(0.128)
n	67	69	69	69
PLAGL1	0.049(0.676)	0.105(0.6355)	0.182(0.123)	-0.054(0.636)
n	76	79	73	79
SNRPN	0.046(0.683)	0.146(0.189)	-0.106(0.345)	-0.05(0.652)
n	82	83	82	85
MEG3	0.116(0.303)	0.054(0.625)	0.028(0.804)	0.185(0.09)
n	81	84	79	85
MTHFR	0.118(0.311)	-0.03(0.793)	0.039(0.733)	0.06(0.598)
n	76	77	78	79
DAZL	0.178(0.15)	0.004(0.971)	-0.006(0.96)	0.01(0.932)
n	67	68	70	70
hNR3C1	-0.008(0.945)	0.17(0.153)	0.012(0.917)	-0.184(0.119)
n	70	72	73	73

Spearman's correlation, ρ, was carried out for global methylation and for each gene or region with BMI, leisure score index (LSI), smoking years, and alcohol consumption. Sig. (*p*) values are shown in brackets.

3.2.1.5.2 Effects of 24 hour exposures on DNA methylation

Although this study focussed on lifestyle and environment in the 3 months prior to the study, exposures in the 24 hours prior to the sample donation were investigated for their potential to confound the methylation results. Men who consumed white meat in the previous 24 hours had a 6.51% higher methylation at *H19* CTCF6 and 0.24% lower methylation at *PLAGL1* (table 3.10). Other gene-specific and global methylation were not altered in response to 24 hour exposures (tables 3.10 and 3.11).

3.2.1.5.3 Effects of lifestyle and health factors on DNA methylation

Lifestyle factors in the 3 months prior to the study were investigated as this is the approximate duration of spermatogenesis, in which time sperm in a

single sample would be affected by these factors. Tables 3.12 and 3.13 list these health and lifestyle factors along with the differences in % DNA methylation at each gene. Those who reported to have a job illness had higher levels of global methylation (413.91 nM) than those who did not (370.3 nM) (p=0.06). Those who reported illness due to stress had higher levels of *MEG3* methylation (89.57%) compared to those who were not stressed (88.2%) (p=0.008). Ex-smokers had higher *SNRPN* methylation (2.3%) than non-smokers (1.96%) (p=0.047). Men who had a diet consisting of meat and fish had higher global methylation (387.74 nM) than those who consumed meat only (328.75 nM) (p=0.039), however the number in the meat-only category was very low (n=5) which questions the validity of this result. DNA methylation at other sites was not affected by these lifestyle factors.

Table 3.10 Imprinted gene methylation and 24-hour exposures

24 hour exposure	n	H19 CTCF6 %	n	H19 exon 1 %	n	PLAGL1 %	n	SNRPN %	n	MEG3 %
Secondary smoke										
Not exposed	80	39.74(36.87-42.61)	61	91.08(90.45-91.7)	72	3.51(3.4-3.63)	73	2.12(1.95-2.32)	76	88.45(88.03-88.83)
Exposed	10	39.03(26.15-51.91)	8	91.83(91.35-92.3)	7	3.81(3.41-4.2)	10	3.04(1.97-5.33)	8	87.8(85.53-89.35)
t-statistic (p-value)		0.157(0.875)		-0.864(0.391)		-1.574(0.12)		1.78(0.105)		-0.929(0.356)
Work attendance										
No attendance	25	42.75(37.2-48.3)	22	90.79(89.42-92.16)	23	3.51(3.3-3.72)	24	2.15(1.93-2.4)	24	88.74(87.83-89.49)
Attendance	65	38.48(35.17-41.78)	47	91.34(90.8-91.88)	56	3.55(3.42-3.68)	60	2.28(2.01-2.6)	62	88.4(87.92-88.84)
t-statistic (p-value)		1.357(0.178)		-0.929(0.356)		-0.319(0.751)		0.725(0.471)		-0.781(0.437)
Red meat consumption										
No	41	37.62(33.11-42.13)	26	91.0 (90.18-91.8)	38	3.62(3.44-3.8)	38	2.11(1.86-2.4)	40	88.61(87.97-89.16)
Yes	49	41.37(37.76-44.98)	43	91.27(90.5-92.03)	41	3.46(3.34-3.59)	46	2.35(2.05-2.71)	46	88.4(87.81-88.92)
t-statistic (p-value)		-1.324(0.189)		-0.486(0.629)		1.428(0.157)		0.237(0.251)		-0.526(0.6)
White meat consumption										
No	56	**37.2(33.68-40.72)**	40	90.9(90.01-91.79)	52	**3.62(3.48-3.77)**	52	2.18(1.94-2.48)	56	88.49(87.94-88.98)
Yes	32	**43.71(39.14-48.29)**	29	91.53(90.99-92.06)	27	**3.38(3.23-3.52)**	33	2.39(2.05-2.82)	29	88.36(87.71-88.93)
t-statistic (p-value)		**-2.297(0.024)***		-1.12(0.267)		**2.216(0.03)***		0.909(0.366)		-0.314(0.754)
Fish consumption										
No	67	40.39(36.94-43.83)	49	91.22(90.53-91.91)	61	3.58(3.46-3.71)	61	2.24(2.01-2.51)	65	88.46(87.97-88.89)
Yes	23	37.56(32.7-42.41)	20	91.03(90.05-92.02)	18	3.38(3.19-3.58)	23	2.21(1.86-2.65)	20	88.41(87.54-89.13)
t-statistic (p-value)		0.870(0.386)		0.297(0.767)		1.563(0.122)		-0.138(0.89)		-0.101(0.919)

Independent t-test t-statistics and p-values are shown. Significant correlations are shown in bold. $p<0.05$*

Table 3.11 Global and gene-specific methylation and 24-hour exposures

24 hour exposure	n	5MedCyd (nM)	n	MTHFR %	n	DAZL %	n	hNR3C1 %
Secondary smoke								
Not exposed	58	373.54(352.68-394.41)	70	1.9(1.80-2.0)	63	2.66(2.33-3.07)	66	5.03(4.82-5.25)
Exposed	7	376.79(327.14-426.44)	9	1.78(1.49-2.08)	5	4.02(2.16-11.1)	7	5.0(4.35-5.73)
t-statistic (p-value)		-0.105(0.917)		0.824(0.412)		1.477(1.44)		0.081(0.936)
Work attendance								
No attendance	18	362.1(335.87-388.34)	23	1.98(1.77-2.18)	17	2.32(1.9-2.9)	18	4.9(4.68-5.13)
Attendance	45	379.93(355.68-404.18)	55	1.86(1.75-1.96)	50	2.84(2.42-3.36)	52	5.02(4.78-5.27)
t-statistic (p-value)		-0.864(0.391)		1.175(0.244)		1.354(0.181)		0.784(0.436)
Red meat consumption								
No	24	381.46(349.34-413.58)	30	1.89(1.75-2.02)	26	2.66(2.25-3.19)	24	4.79(4.52-5.06)
Yes	35	381.00(359.68-402.32)	49	1.89(1.77-2.02)	42	2.78(2.31-3.42)	44	5.08(4.83-5.35)
t-statistic (p-value)		0.025(0.98)		-0.051(0.959)		0.322(0.748)		-1.529(0.131)
White meat consumption								
No	30	394.85(371.78-417.92)	46	1.94(1.81-2.06)	38	2.59(2.25-3.0)	44	4.95(4.71-5.2)
Yes	29	367.06(339.97-394.14)	33	1.82(1.68-1.97)	27	2.87(2.28-3.71)	27	5.0(4.69-5.32)
t-statistic (p-value)		1.603(0.114)		1.199(0.234)		0.761(0.451)		-0.242(0.809)
Fish consumption								
No	43	375.93(354.30-397.57)	57	1.88(1.76-1.99)	51	2.82(2.42-3.32)	53	5.01(4.8-5.23)
Yes	16	395.32(363.91-426.72)	22	1.93(1.76-2.09)	17	2.51(1.92-3.42)	15	4.95(4.68-5.23)
t-statistic (p-value)		-0.981(0.331)		0.577(0.61)		-0.743(0.46)		0.355(0.725)

Independent t-test t-statistics and *p*-values are shown.

Lifestyle factor	n	H19 CTCF6 %	n	H19 exon 1 %	n	PLAGL1 %	n	SNRPN %	n	MEG3 %
Fever or flu										
No	78	39.3(36.15-42.45)	59	91.2(90.55-91.83)	68	3.52(3.4-3.63)	72	2.14 (1.96-2.35)	73	88.47(88.04-88.86)
Yes	13	41.15(31.9-50.41)	10	91.0(90.37-91.63)	11	3.36(3.3-4.02)	13	2.17(1.64-2.96)	13	87.93(86.51-89.02)
t-statistic (p-value)		-0.431(0.667)		0.236(0.814)		-0.892(0.375)		-0.087(0.931)		-0.976(0.332)
Job illness										
No	74	40.2(37.13-43.27)	57	91.13(90.47-91.8)	66	3.53(3.41-3.65)	69	1.99(1.85-2.14)	70	88.22(87.71-88.69)
Yes	16	36.93(29.28-44.57)	12	91.3(90.73-91.88)	13	3.57(3.32-3.82)	16	1.85(1.63-2.11)	15	88.83(88.05-89.48)
t-statistic (p-value)		0.884(0.379)		-0.233(0.817)		-0.286(0.776)		-0.864(0.391)		1.162(0.248)
Illness due to stress										
No	81	40.09(37.12-43.05)	62	91.17(90.56-91.78)	72	3.53(3.41-3.65)	76	2.13(1.96-2.33)	74	**88.2(87.75-88.61)**
Yes	10	36.25(26.79-45.71)	7	91.09(90.08-92.1)	7	3.61(3.3-3.93)	8	1.85(1.54-2.24)	10	**89.57(88.92-90.11)**
t-statistic(p-value)		0.856(0.394)		0.088(0.93)		-0.438(0.663)		-1.072(0.287)		**2.714(0.008)****
Smoking history										
Non-smoker	48	39.8(35.57-44.22)	37	91.27(90.6-91.95)	45	3.54(3.4-3.68)	39	**1.96(1.82-2.12)**	44	88.17(87.57-88.69)
Ex-smoker	41	39.55(35.81-43.28)	31	91.0(90.03-91.97)	32	3.57(3.4-3.74)	39	**2.3(2.0-2.65)**	38	88.59(87.98-89.12)
t-statistic (p-value)		0.121(0.904)		0.484(0.63)		-0.278(0.782)		**2.031(0.047)***		1.068(0.289)
Alcohol consumption										
<1 drink per week	27	39.51(34.41-44.6)	21	91.75(91.24-92.27)	24	3.52(3.31-3.72)	27	2.18(1.79-2.7)	25	88.8(88.13-89.37)
>1 drink per week	64	39.74(36.32-43.15)	48	90.9(90.14-91.67)	55	3.55(3.42-3.68)	58	2.24(2.04-2.48)	60	88.29(87.76-88.75)
t-statistic (p-value)		-0.074(0.941)		1.422(0.16)		-0.255(0.799)		0.296(0.768)		-1.227(0.223)
Underwear										
No boxer shorts	7	40.29(34.87-45.71)	4	88.7(76.97-100.43)	6	3.55(2.88-4.23)	6	2.30(1.37-4.43)	7	88.56(85.43-90.34)
Boxer shorts	82	39.26(36.25-42.28)	64	91.31(90.9-91.73)	72	3.54(3.43-3.65)	77	2.23(2.03-2.46)	77	88.43(88.01-88.81)
t-statistic (p-value)		-0.38(0.71)		-0.708(0.53)		0.052(0.959)		-0.159(0.874)		-0.184(0.855)
Leisure score index										
<24 points	50	38.52(35.09-41.95)	39	90.86(89.96-91.76)	43	3.46(3.3-3.61)	48	2.37(2.1-2.69)	45	**88.78(88.3-89.2)**
≥24 points	39	40.44(35.65-45.23)	30	91.56(91.02-92.09)	36	3.63(3.48-3.78)	35	2.05(1.77-2.39)	37	**88.14(87.48-88.72)**
t-statistic (p-value)		-0.674(0.502)		-1.248(0.216)		-1.622(0.109)		-1.533(0.129)		**-1.717(0.09)**
BMI (kg/m^2)										
<25	44	41.56(37.58-45.54)	36	91.26(90.77-91.76)	40	3.54(3.4-3.68)	43	2.12(1.89-2.4)	36	88.6(88.0-89.13)
25-29.9	28	37.39(33.34-41.44)	19	91.03(89.97-92.08)	25	3.6(3.4-3.8)	24	2.29(1.98-2.67)	22	90.61(87.87-89.09)
≥30	13	38.86(29.22-48.5)	12	90.9(88.24-93.57)	11	3.42(3.1-3.73)	12	1.98(1.48-2.72)	11	87.68(86.07-88.91)
F-statistic (p-value)		0.953(0.39)		0.132(0.877)		0.608(0.547)		0.578(0.563)		1.198(0.307)
Diet										
Meat eater	15	39.96(33.11-46.81)	6	91.54(91.32-91.76)	15	3.66(3.27-4.05)	12	1.85(1.44-2.44)	14	87.96 (86.81-88.88)
Meat and fish eater	74	39.69(36.49-42.87)	62	91.17(90.56-91.78)	63	3.5(3.4-3.6)	70	2.28(2.07-2.52)	69	88.43(87.98-88.83)
t-statistic (p-value)		0.072(0.943)		0.373(0.711)		1.157(0.251)		1.641(0.105)		0.867(0.388)

Table 3.12 Imprinted gene methylation and lifestyle factors. Independent t-test t-statistics and *p*-values are shown. Correlations with *p*-value <0.1 are shown in bold. *p<0.05, **p<0.01.

Lifestyle factor	n	5MedCyd (nM)	n	MTHFR %	n	DAZL %	n	hNR3C1 %
Fever or flu								
No	52	375.83(355.16-396.68)	68	1.88(1.78-1.98)	60	2.82(2.45-3.29)	61	5.04(4.87-5.22)
Yes	10	389.55(360.15-418.95)	11	1.96(1.7-2.22)	7	2.48(2.08-3.01)	9	4.45(3.87-5.78)
t-statistic (p-value)		-0.565(0.574)		-0.606(0.546)		-1.215(0.239)		1.056(0.295)
Job illness								
No	51	**370.3(349.83-390.78)**	63	1.87(1.76-1.97)	55	2.73(2.36-3.19)	60	5.1(4.88-5.34)
Yes	11	**413.91(387.25-440.57)**	15	1.99(1.77-2.21)	13	2.78(2.03-4.02)	12	4.73(4.24-5.27)
t-statistic (p-value)		**-1.918(0.06)**		-1.041(0.301)		0.109(0.914)		1.38(0.172)
Illness due to stress								
No	55	375.21(355.43-394.98)	69	1.89(1.78-1.99)	61	2.67(2.33-3.08)	61	4.97(4.78-5.16)
Yes	7	400.31(373.51-427.10)	10	1.93(1.69-2.5)	9	3.01(1.94-5.29)	8	4.99(4.34-5.71)
t-statistic(p-value)		-0.894(0.375)		-0.296(0.768)		0.654(0.515)		-0.064(0.949)
Smoking history								
Non-smoker	35	384.21(360.25-408.17)	40	1.71(1.6-1.82)	42	1.89(1.77-2.0)	40	5.05(4.73-5.38)
Ex-smoker	25	377.75(351.46-404.04)	34	1.79(1.66-1.92)	35	1.91(1.75-2.06)	30	5.09(4.86-5.32)
t-statistic (p-value)		0.366(0.715)		-0.969(0.336)		-0.176(0.861)		-0.188(0.851)
Alcohol consumption								
<1 drink per week	17	387.65(361.29-414.0)	22	1.8(1.62-1.98)	19	2.64(2.1-3.39)	21	5.07(4.7-5.47)
>1 drink per week	43	382.59(361.95-403.24)	57	1.92(1.81-2.04)	49	2.78(2.36-3.31)	52	5.01(4.77-5.26)
t-statistic (p-value)		0.279(0.781)		-1.201(0.233)		0.348(0.729)		-0.269(0.789)
Underwear								
No boxer shorts	4	348.16(301.61-394.7)	6	1.88(1.59-2.16)	4	3.84(1.02-11.18)	6	4.97(3.95-6.19)
Boxer shorts	57	379.2(360.14-398.25)	71	1.89(1.79-1.99)	62	2.71(2.37-3.11)	66	5.03(4.82-5.24)
t-statistic (p-value)		-0.854(0.397)		-0.073(0.942)		-0.691(0.538)		-0.164(0.87)
Leisure score index								
<24 points	33	376.2(350.8-401.6)	44	1.74(1.64-1.86)	39	2.87(2.41-3.47)	37	4.99(4.77-5.22)
≥24 points	29	380.14(353.88-406.4)	28	1.64(1.54-1.73)	28	2.57(2.08-3.24)	33	5.1(4.77-5.44)
t-statistic (p-value)		-0.914(0.827)		1.497(0.139)		-0.798(0.428)		-0.536(0.594)
BMI (kg/m^2)								
<25	31	369.71(346.37-393.05)	41	1.86(1.74-1.98)	33	2.94(2.39-3.7)	34	4.97(4.71-5.25)
25-25.99	17	401.67(361.27-442.07)	23	1.87(1.69-2.05)	32	2.32(1.95-2.81)	22	5.15(4.77-5.55)
≥30	10	375.0(334.58-416.63)	11	2.16(1.84-2.48)		2.47(1.9-3.32)	11	4.93(4.53-5.37)
F-statistic		1.05(0.376)		2.463(0.092)		1.483(0.235)		0.392(0.759)
Diet								
Meat eater	5	**328.75(241.4-416.09)**	11	**1.66(1.35-1.98)**	10	2.94(2.06-4.55)	10	4.93(4.59-5.28)
Meat and fish eater	53	**387.74(370.56-404.91)**	67	**1.93(1.83-2.03)**	57	2.75(2.38-3.2)	59	5.06(4.84-5.29)
t-statistic (p-value)		**-2.112(0.039)***		**-1.998(0.049)***		-0.361(0.719)		-0.479(0.634)

Table 3.13 Global and non-imprinted gene methylation and lifestyle factors. Independent t-test t-statistics and *p*-values are shown. Correlations with *p*-value <0.1 are shown in bold. *p<0.05, **p<0.01.

Exposure	n	H19 CTCF6 %	n	H19 exon 1 %	n	PLAGL1 %	n	SNRPN %	n	MEG3 %
Metal dust or fumes										
No	70	39.43(36.21-42.65)	54	90.91(90.22-91.4)	64	3.5(3.39-3.64)	66	2.27(2.06-2.51)	64	88.57(88.16-88.95)
Yes	18	41.79(35.02-48.57)	13	92.0(91.48-92.52)	13	3.66(3.36-3.96)	16	2.13(1.59-2.93)	17	88.25(87.13-89.15)
t-statistic (p-value)		-0.662(0.51)		-1.534(0.13)		-0.954(0.343)		-0.428(0.673)		-0.691(0.492)
Oils or greases										
No	63	38.77(35.55-42.0)	49	90.93(90.17-91.69)	57	3.5(3.38-3.62)	61	2.34(2.08-2.65)	60	88.51(87.98-88.97)
Yes	25	42.78(36.61-48.96)	18	91.64(91.17-92.11)	20	3.64(3.37-3.91)	20	2.06(1.81-2.36)	24	88.7(88.02-89.28)
t-statistic (p-value)		-1.263(0.21)		-1.103(0.274)		-1.044(0.3)		-1.454(0.152)		0.446(0.657)
Detergents and soaps										
No	55	38.35(34.58-42.11)	40	91.12(90.52-91.72)	50	3.58(3.44-3.71)	53	2.17(1.94-2.45)	52	**88.75(88.29-89.17)**
Yes	33	42.52(38.09-46.95)	27	91.12(89.97-92.27)	27	3.47(3.27-3.67)	30	2.46(2.06-2.98)	31	**88.07(88.27-88.75)**
t-statistic (p-value)		-1.414(0.161)		0.0(1.0)		0.914(0.363)		1.203(0.232)		**-1.707(0.092)**
Glues, adhesives, resins										
No	62	39.23(35.87-42.59)	50	**90.85(90.11-91.58)**	54	3.52(3.38-3.65)	59	**2.39(2.13-2.71)**	57	88.52(88.08-88.92)
Yes	26	41.54(35.79-47.28)	17	**91.93(91.47-92.39)**	23	3.59(3.39-3.8)	23	**1.82(1.63-2.05)**	24	88.47(87.64-89.16)
t-statistic (p-value)		-0.731(0.467)		**-1.687(0.096)**		-0.652(0.516)		**-3.328(0.001)****		-0.129(0.898)
Paints, varnishes, lacquers										
No	65	**38.48(35.15-41.81)**	51	90.89(90.18-91.61)	56	3.52(3.38-3.65)	63	2.26(2.03-2.53)	61	88.58(88.08-89.02)
Yes	23	**43.95(38.29-49.62)**	16	91.84(91.18-92.5)	21	3.59(3.35-3.82)	20	2.29(1.84-2.9)	23	88.53(87.73-89.19)
t-statistic (p-value)		**-1.69(0.095)**		-1.426 (0.159)		-0.526(0.601)		0.102(0.919)		-0.114(0.91)
Other solvents										
No	65	39.44(36.22-42.66)	50	90.92(90.17-91.66)	60	3.5(3.37-3.63)	63	2.18(1.97-2.43)	62	88.74(88.31-89.13)
Yes	23	41.23(34.75-47.72)	17	91.73(91.28-92.18)	17	3.68(3.46-3.91)	20	2.7(2.03-3.35)	21	87.97(86.84-88.89)
t-statistic (p-value)		-0.545(0.587)		-1.258(0.213)		-1.384(0.17)		1.406(0.163)		-1.651(0.103)
Heavy vib. machinery										
No	74	39.54(36.42-42.65)	58	90.97(90.32-91.61)	66	**3.5(3.38-3.62)**	70	2.27(2.06-2.52)	71	88.5(88.05-88.91)
Yes	14	41.9(33.77-50.03)	9	92.14(91.56-92.71)	11	**3.79(3.5-4.09)**	13	2.24(1.6-3.32)	13	88.89(87.77-89.75)
t-statistic (p-value)		-0.599(0.55)		-1.415(0.162)		**-1.91(0.06)**		-0.076(0.94)		0.739(0.462)

Table 3.14 Imprinted gene methylation and chemical and physical exposures. Independent t-test t-statistics and *p*-values are shown. Correlations with *p*-value <0.1 are shown in bold. *p<0.05, **p<0.01.

Exposure	n	5MedCyd (nM)	n	MTHFR %	n	DAZL %	n	hNR3C1 %
Metal dust or fumes								
No	48	382.51(363.68-401.34)	60	1.89(1.79-2.0)	52	**2.59(2.24-3.01)**	53	5.01(4.83-5.21)
Yes	11	375.43(320.83-430.03)	17	1.85(1.61-2.09)	14	**3.57(2.5-5.6)**	15	4.77(4.31-5.28)
t-statistic (p-value)		0.27(0.791)		-0.345(0.731)		**1.845(0.07)**		1.116(0.268)
Oils or greases								
No	44	375.69(357.89-393.48)	55	1.91(1.81-2.02)	47	2.72(2.32-3.24)	50	5.05(4.83-5.27)
Yes	15	397.34(347.9-446.76)	22	1.81(1.6-2.01)	19	2.85(2.22-3.81)	19	4.83(4.47-5.2)
t-statistic (p-value)		-0.877(0.392)		1.053(0.296)		0.305(0.761)		1.079(0.284)
Detergents and soaps								
No	37	373.15(347.85-398.44)	46	1.93(1.81-2.04)	38	2.62(2.18-3.21)	40	5.0(4.76-5.26)
Yes	23	386.08(413.48-413.48)	31	1.82(1.65-1.98)	25	2.62(2.25-3.09)	28	4.9(4.64-5.17)
t-statistic (p-value)		-0.683(0.498)		1.19(0.238)		-0.003(0.997)		0.595(0.554)
Glues, adhesives, resins								
No	43	**371.37(354.53-388.21)**	56	1.9(1.8-2.0)	48	2.79(2.36-3.36)	49	4.99(4.78-5.2)
Yes	14	**412.2(372.99-451.42)**	21	1.85(1.64-2.06)	18	2.67(2.15-3.4)	20	4.98(4.58-5.41)
t-statistic (p-value)		**-2.283(0.026)***		0.445(0.658)		-0.296(0.768)		0.031(0.976)
Paints, varnishes, lacquers								
No	44	374.5(354.49-394.51)	57	1.88(1.78-1.98)	50	2.69(2.29-3.2)	51	4.9(4.72-5.09)
Yes	15	400.82(361.63-440.0)	20	1.9(1.67-2.13)	16	3.0(2.38-3.9)	18	5.22(4.72-5.76)
t-statistic (p-value)		-1.313(0.195)		-0.196(0.846)		0.671(0.505)		-1.488(0.142)
Other solvents								
No	44	372.85(354.26-391.43)	56	1.93(1.82-2.03)	51	2.68(2.3-3.16)	52	4.97(4.76-5.19)
Yes	15	405.66(360.92-450.4)	21	1.77(1.57-1.93)	15	3.04(2.26-4.29)	16	4.92(4.57-5.29)
t-statistic (p-value)		-1.65(0.104)		1.447(0.152)		0.731(0.468)		0.238(0.813)
Heavy vib. machinery								
No	51	380.16(360.3-400.01)	65	1.91(1.81-2.02)	57	2.78(2.39-3.26)	58	4.98(4.79-5.18)
Yes	8	387.77(349.67-425.86)	12	1.73(1.52-)-	9	2.66(1.89-3.99)	11	5.0(4.4-5.68)
t-statistic (p-value)		-0.294(0.77)		0.284(0.777)		-0.203(0.84)		-0.098(0.923)

Table 3.15 Global and non-imprinted gene methylation and chemical and physical exposures
Independent t-test t-statistics and *p*-values are shown. Correlations with *p*-value <0.1 are shown in bold.

3.2.1.5.4. Effects of chemical and physical exposures on DNA methylation

Men were asked about whether they were exposed to certain chemical and physical hazards at home or at work. Men who were exposed to glues, adhesives or resins had significantly lower *SNRPN* methylation (*p*=0.001), significantly higher global methylation (*p*=0.026) (table 3.14 and 3.15). A multivariate analysis was then performed with each of these variables. Exposures which showed an association with DNA methylation with a *p*-value less than 0.1 were also included in the multivariate analysis. These included heavy vibrating machinery and *PLAGL1* methylation (*p*=0.06), metal dust or fumes and *DAZL* methylation (*p*=0.07), and, finally, detergents and MEG3 methylation (*p*=0.09). *MTHFR*, *NR3C1* and *H19* CTCF6 did not show any associations with chemical or physical exposures (*p*>0.1).

Table 3.16 Multivariate analysis of the effects of lifestyle and environmental exposures on DNA methylation

Model	DV	IV	Unstandardised B-coefficient	Standardised β-coefficient	Sig. (p-value)	ANOVA (p-value)
1	[a]5MedCyd (nM)	Glues, adhesives, resins	60.859	0.323	**0.019***	0.076
2	[a]*MEG3* %	Stress	-0.096	-0.132	0.297	0.66
		Detergents/soaps	0.043	0.096	0.435	
		LSI	0.001	0.094	0.481	
3	[a]*SNRPN* %	Smoking (non-/ex-)	-0.044	-0.231	**0.052**	0.08
		Glues, adhesives, resins	0.036	0.178	0.137	
4	[a]*DAZL* %	Metal dust or fumes	-0.064	-0.211	0.108	0.144
5	[b]*H19* CTCF6 %	Paints, varnishes, lacquers	5.148	0.164	0.149	0.112
6	[a]*MTHFR* %	Diet	0.013	0.011	0.934	0.718
7	[b]*PLAGL1* %	Heavy vibrating machinery	-0.145	-0.295	0.769	0.363
8	[a]*H19* exon 1 %	Glues, adhesives, resins	1.273	0.253	**0.061**	0.077

Each row represents a different linear regression model showing the effect of listed IVs on DVs while controlling for age, BMI and abstinence time ([a]) and 24 hour white meat consumption ([b]). Sig. (p-values) are shown for each IV. Control variables, not listed, did not show any significance *(p*-value>0.05) except white meat which was linked to *H19* exon 1(*p*=0.038). ANOVA *p*-values show the overall significance of the model as a good predictor of DV outcome considering all IVs.

3.2.1.5.5. Multivariate analysis of lifestyle and environmental exposures on DNA methylation

To confirm whether the exposures in the univariate analysis were significant when controlling for other factors, multiple linear regression models were carried out controlling for age, BMI and abstinence time. Although these control variables were not found to be associated with methylation independently, they may influence the trends of other exposures, and were therefore included as independent variables (IV).

The results showed that exposure to glues, adhesives and resins was still significantly associated with an increase in global DNA methylation (p=0.019), but it was no longer linked to a change in *SNRPN* methylation (p=0.137). The remaining chemical and physical exposures did not show significant associations with DNA methylation.

Table 3.17 DNA methylation correlation matrix

	H19 CTCF6 %	H19 exon 1 %	MEG3 %	PLAGL1 %	SNRPN %	MTHFR %	DAZL %	NR3C1 %
5MedCyd (nM)								
ρ	0.006	0.008	-0.045	0.244	-0.19	0.066	-0.186	0.07
p-value	0.96	0.948	0.723	0.061	0.136	0.61	0.183	0.593
N	66	62	62	60	63	62	53	61
H19 CTCF6 %								
ρ		0.162	-0.043	**-0.245***	0.024	0.032	-0.135	-0.098
p-value		0.188	0.188	**0.03**	0.827	0.779	0.266	0.408
n		68	87	**78**	87	79	70	74
H19 exon 1 %								
ρ			-0.031	-0.123	-0.033	-0.048	-0.044	0.112
p-value			0.804	0.344	0.793	0.707	0.747	0.383
n			65	61	65	64	56	63
MEG3 %								
ρ				0.056	-0.095	0.143	0.043	-0.083
p-value				0.623	0.399	0.225	0.729	0.485
n				78	81	74	67	73
PLAGL1 %								
ρ					-0.196	-0.001	**-0.286***	0.187
p-value					0.097	0.993	**0.028**	0.129
n					73	67	**59**	67
SNRPN %								
ρ						-0.066	**0.358****	0.056
p-value						0.572	**0.003**	0.643
n						76	**67**	71
MTHFR %								
ρ							0.031	**0.293***
p-value							0.804	**0.015**
n							67	**69**
DAZL %								
ρ								0.225
p-value								0.069
n								66

Sig. (*p*) values for Spearman's correlation, *ρ*, between global methylation and gene-specific methylation. Significant correlations are shown in bold. **p*<0.05, ***p*<0.01. The direction of the correlations for *SNRPN* and *DAZL* have been reversed as the reciprocal transformation had been used for these data sets.

3.2.1.6 Comparisons and trends in DNA methylation between genes

Alterations in DNA methylation can occur at single sites or affect multiple genes. This can result in two or more genes showing similar patterns of increasing or decreasing methylation, or genes may show opposing effects, i.e. higher methylation at one site can be linked to lower methylation at

another. Furthermore, a marker of aberrant methylation processes is the disruption of paternal and maternal imprints, so paternally imprinted genes may become demethylated while maternally imprinted genes become methylated in sperm. Correlation analyses between genes and between genes and global methylation were carried out to determine whether there were associations between those under consideration in this study (table 3.17).

Bivariate correlations between global methylation and each gene showed that global methylation was not significantly associated with gene-specific methylation.

Methylation of maternally imprinted *PLAGL1* was negatively correlated with paternally imprinted *H19* CTCF6 (p=0.03) and also negatively correlated with *DAZL* methylation (p=0.028) (fig. 3.6 B and D). *DAZL* methylation was highly positively correlated with *SNRPN* methylation (p=0.003) (fig. 3.6 C). Finally, the gene with a critical role in the methylation of DNA, *MTHFR,* was positively correlated with the *NR3C1* methylation (p=0.015) (fig. 3.6 A).

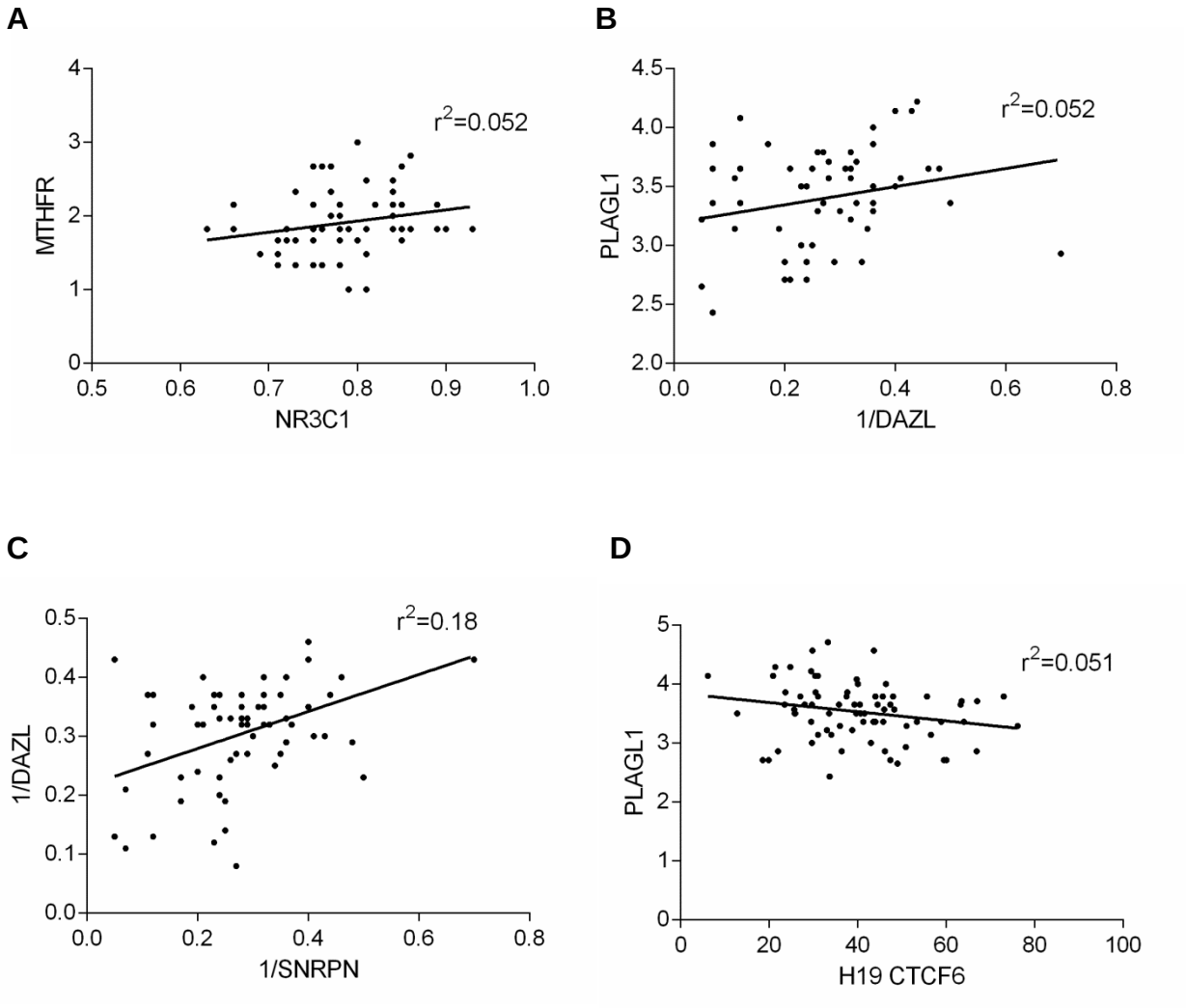


Figure 3.6 Gene correlations

Graphical representation of gene methylation correlations showing goodness of fit, r^2, values. Correlations were made using transformed data so the real trends for *DAZL* and *SNRPN* are the reverse of that shown, so *DAZL* is negatively correlated with *PLAGL1*.

3.2.2 Sperm DNA methylation and semen parameters

3.2.2.1 Normalisation of semen parameter data

Semen parameters were collected from 94 men and consisted of sperm concentration, sperm motility grades (i.e. A, B, C and D) and semen volume. The different motility grades were categorised as % progressive motility (PM) (grade A and B) and % immotile cells (grade D) and total sperm count was calculated from the concentration and volume. Table 3.18 shows the means, standard deviation (SD), minimum and maximum values, 25th and 75th percentiles and 95% confidence intervals for the raw data and following transformation (see section 2.1.2 for further explanation of data transformations).

Table 3.18 Original and transformed semen parameters data

Semen parameters	n	Mean(SD)	Min	Max	25th percentile	75th percentile	95% CI	
							Lower	Upper
Concentration (x10^6/ml)	94	70.6(63.1)	0.5	362.0	29.25	148.5	57.7	83.6
PM (%)	94	51.7(13.8)	12.0	83.0	42.75	60.3	48.9	54.5
Immotile (%)	94	39.1(12.4)	13.0	73.0	30.0	47.3	36.6	41.6
Volume (ml)	93	3.2(1.5)	0.6	8.1	2.0	3.9	2.5	3.5
Count (x10^6)	93	210.7(176.3)	0.7	840.0	64.5	320.5	174.4	247.0
Transformed parameters	n	Geometric mean	Min	Max	25th percentile	75th percentile	95% CI	
							Lower	Upper
Log10 concentration	94	48.6	0.51	362.1	30.6	95.7	38.6	58.7
Log10 count	91	126.6	0.7	601.6	63.0	290.47	99.3	161.3

The top section of the table illustrates the original semen parameter data while the bottom section shows the same parameters after transformation and/or outlier removal. Means for transformed data are geometric means and 95% confidence intervals and percentiles and min/max values were back-transformed from the log^{10} data.

3.2.2.2 DNA methylation and sperm parameters

3.2.2.2.1 Univariate analysis of DNA methylation and sperm parameters

To investigate the association between semen parameters and DNA methylation, correlations were made for each parameter and each gene or global methylation. The analysis revealed that global DNA methylation was negatively correlated with % PM (p=0.016) and positively correlated with % immotile cells (p=0.005) (fig. 3.7 A and B). *DAZL* methylation was highly

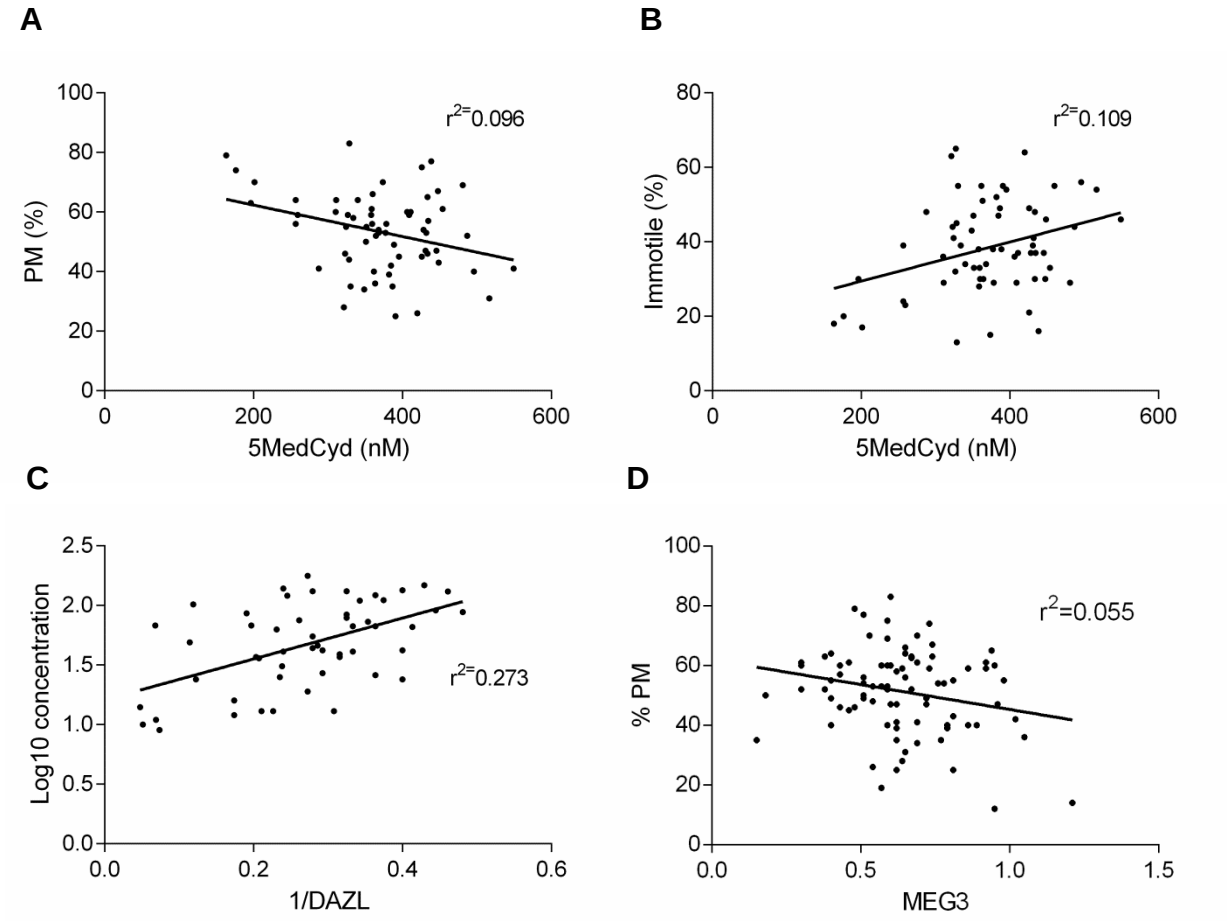


Figure 3.7 DNA methylation and semen parameters correlations

Global methylation was negatively correlated with % progressive motility (A) and positively correlated with % immotile cells (B). 1/DAZL was positively correlated with sperm concentration (p<0.001) (C). *MEG3* was negatively correlated with % progressive motility (D).

correlated with sperm concentration (p<0.001) and sperm count (p=0.005) (fig. 3.7 C). As the reciprocal transformation of *DAZL* $(1/DAZL)$ was used for data analysis, the correlation shown in the graph is in fact negative: higher *DAZL* methylation is linked to lower sperm concentration and sperm count. Univariate analysis showed that *MEG3* methylation was negatively correlated with % PM (p=0.023) and that *SNRPN* methylation was positively correlated with sperm count (p=0.048).

When sperm parameters within the WHO threshold for normality were analysed, the positive correlation between global methylation and % immotile sperm was still significant (p=0.028) and the positive correlation between *DAZL* methylation and sperm concentration was also still highly significant (p=0.009) (appendix 13, table 2). DNA methylation of *H19* CTCF6, *H19* exon 1, *PLAGL1*, *SNRPN*, *MEG3*, *MTHFR*, and *NR3C1* were not correlated with any semen parameters in men within the WHO threshold for normality (appendix 13, table 1).

3.2.2.2.2 Multivariate analysis of DNA methylation and sperm parameters

To confirm the associations between DNA methylation and sperm parameters found in the univariate models while controlling confounding

Table 3.19 Multivariate analysis of DNA methylation and sperm parameters

Model	DV	IV	Unstandardised B-coefficient	Standardised β-coefficient	Sig (p-value)	ANOVA (p-value)
1	% PM	5mC (nM)	-0.053	-0.277	**0.043***	0.091
2	% immotile	5mC (nM)	0.051	0.318	**0.017***	**0.018***
3	Conc. (x10^6/ml)	*DAZL %*	1.606	0.416	**0.001****	**0.005****
4	% PM	*MEG3 %*	-17.633	-0.266	**0.024***	0.115

Each row represents a different multiple linear regression model showing the effect of DNA methylation (IV) on sperm parameters (DV) while controlling for age, BMI and abstinence time. Sig. (p-values) are shown for each IV. Control variables, not listed, did not show any significance *(*p-value>0.05). ANOVA p-values show the overall significance of the model.

factors, multiple regression analyses were carried out with age, BMI and abstinence time as additional IVs. All genes that showed significance <0.1 were included in the analysis. The results showed that increasing global DNA methylation is still significantly positively associated with % immotile sperm and negatively associated with % PM (table 3.19). Standardised β-coefficients, which represent the relative change in the DV based on each IV, show that changes in global methylation are more closely linked to changes in % immotile sperm (p=0.017) than in % PM (p=0.043) as there is a larger coefficient value. 1/*DAZL* methylation was still significantly highly correlated with sperm concentration (p=0.001); so increasing *DAZL* methylation is linked to lower sperm concentration. Finally, *MEG3* methylation was negatively correlated with % PM (p=0.024).

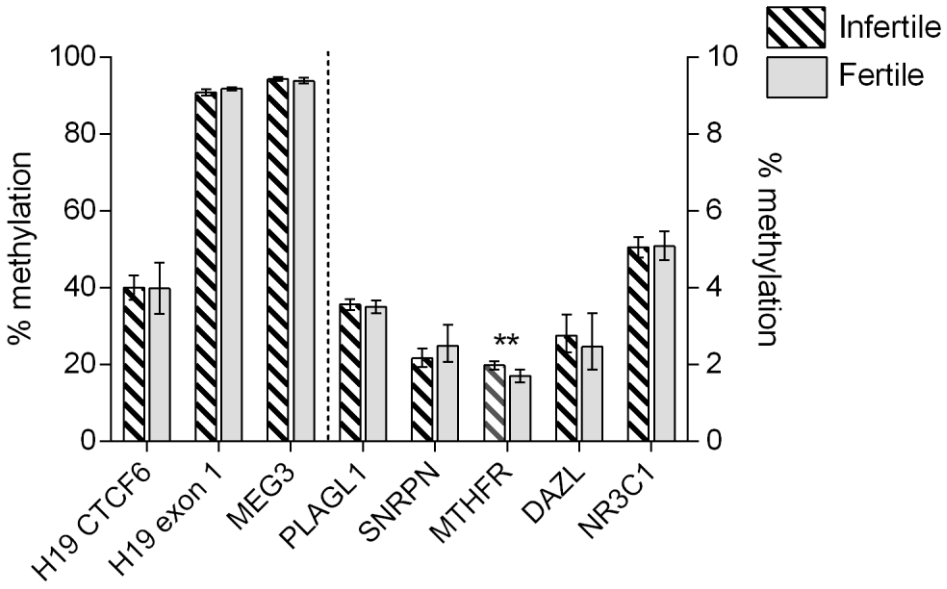


Figure 3.8 DNA methylation in fertile and infertile men

Independent t-tests were carried out between fertile and infertile men. *MTHFR* showed higher methylation in the infertile group **p<0.001. Data sets either side of the dotted line are plotted on the left and right y-axes. Error bars represent 95% CI.

3.2.2.3. DNA methylation in fertile and infertile men

Following consultation and semen analysis at the fertility hospital, men were classed as either fertile or infertile based on their semen parameters or on failed previous fertility. Some men classed as infertile had semen parameters in the normal range, suggesting that the cause of their infertility may be due to factors other than the standard observable semen parameters. Therefore, DNA methylation was compared between these two groups of men (fig. 3.8). *MTHFR* methylation was higher in the sperm of infertile men compared with fertile men (p=0.006). No other genes or global methylation showed a significant difference in methylation.

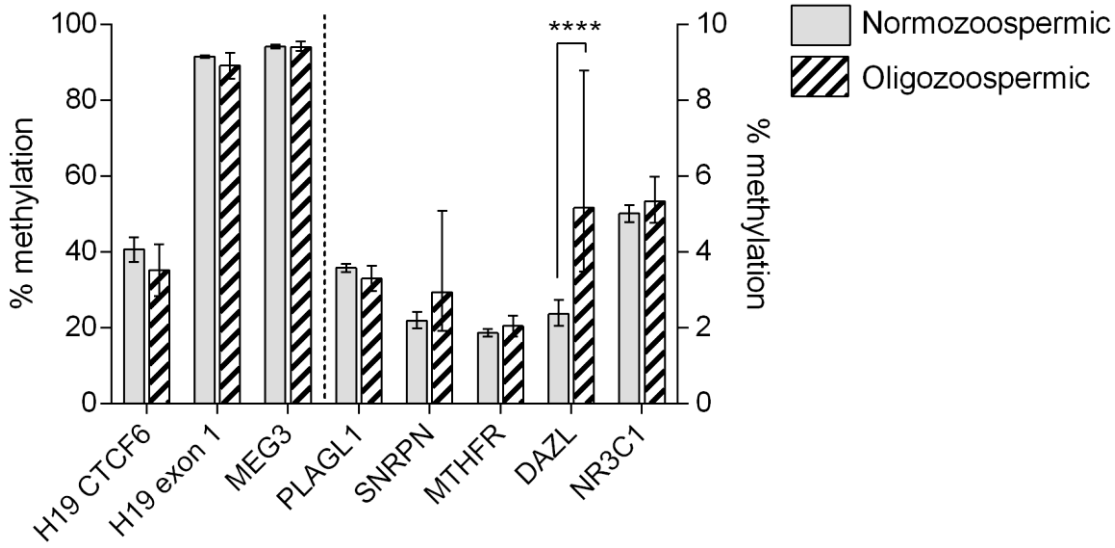


Figure 3.9 DNA methylation in normozoospermic and oligozoospermic men

Independent t-tests were carried out between the two groups. *DAZL* showed higher methylation in the oligozoospermic group ****p<0.0001. Data sets either side of the dotted line are plotted on the left and right y-axes. Error bars represent 95% CI.

3.2.2.4 DNA methylation in oligozoospermic men

Men with sperm count less than $15x10^6$/ml are classed as oligozoospermic. To identify whether this subgroup of men have different methylation levels to men with normal sperm concentration, DNA methylation was investigated in men with oligozoospermia (n=72) compared to men with normal semen parameters (n=12). The numbers of oligozoospermic men were low overall therefore consideration was taken to prevent false positive results (see appendix 13, table 4).

DAZL was the only gene to show a significant difference between the oligozoospermic and normozoospermic groups, having methylation values of 5.16% and 2.36%, respectively. The oligozoospermic group contained the two highest methylation values in the data set: 18.5% and 20% methylation which had concentrations of $9x10^6$ and $13x10^6$ cells/ml, respectively.

3.2.2.5 DNA methylation in whole and prepared sperm

During the preparation of sperm for ART, motile and mature sperm are separated from immature non-motile sperm, cellular debris and non-sperm cells using density gradient centrifugation. A fundamental question of this process is whether the purified sperm population is distinct at the epigenetic level from the whole sperm population as the former is subsequently used for IVF or ICSI. To investigate this, DNA methylation was compared between whole sperm, obtained from neat semen washed in PBS only, and that of prepared sperm, which had undergone density gradient centrifugation using preparation media.

DNA methylation was significantly higher in prepared sperm than in whole semen for all the imprinted genes including *H19* exon 1 ($p<0.0001$), *PLAGL1* ($p=0.0052$) and *PEG10* ($p=0.0062$), as well as for *MTHFR* ($p=0.037$) (fig. 3.10 B). Global DNA methylation was also significanly higher in prepared sperm with a mean of 502.4 nM (±43.42) compared with whole sperm at 369.8 nM (±39.97) ($p=0.016$) (fig. 3.10 A).

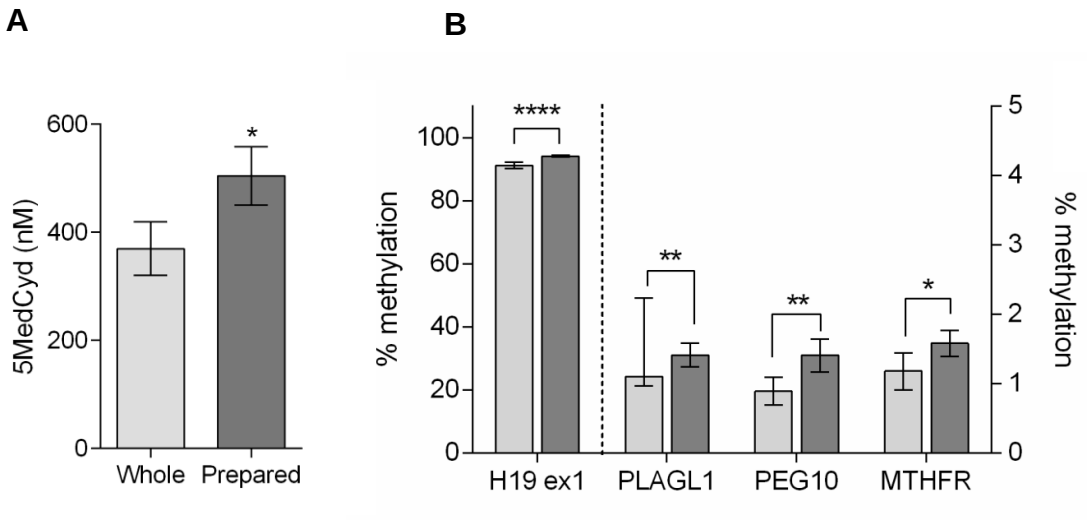


Figure 3.10 DNA methylation in whole and prepared sperm

Global methylation (n=4) (A) and methylation of the imprinted genes *H19, PLAGL1* and *PEG10* and *MTHFR* (B) in whole sperm (light grey bars) and prepared sperm (dark grey bars). Independent t-tests between whole and prepared sperm were carried out. Data sets either side of the dotted line (B) are plotted on the left and right y-axes. Error bars represent upper and lower 95% Cis. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

3.2.2.6 DNA methylation as a marker of WBC contaminants in whole sperm

White blood cell (WBC) contamination in semen often occurs due to infection, and high numbers of WBCs (leukocytospermia) can cause infertility. Clinical sperm preparation should remove these contaminating cells. Two genes that are specifically expressed and non-methylated in WBCs are *CD247* and *LSP1.* These genes should show hypermethylation, and therefore low/no expression, in sperm. Methylation of these genes was therefore investigated as a potential marker for WBC contamination in whole sperm, as this should show lower methylation levels than prepared sperm.

The results showed that *CD247* was in fact hypomethylated in sperm (fig. 3.11 A) and furthermore, methylation levels were significantly lower in prepared than whole sperm. This contrasts the trend seen with all other genes and global methylation which show higher methylation in prepared sperm (fig. 3.10).

LSP1 showed a hypermethylated state that was expected of this WBC marker in sperm, however no significant difference was found between *LSP1* methylation in whole and prepared sperm (fig. 3.11 B). WBC counts were not available but would have confirmed whether the change in *CD247* and *LSP1* were related to WBC numbers.

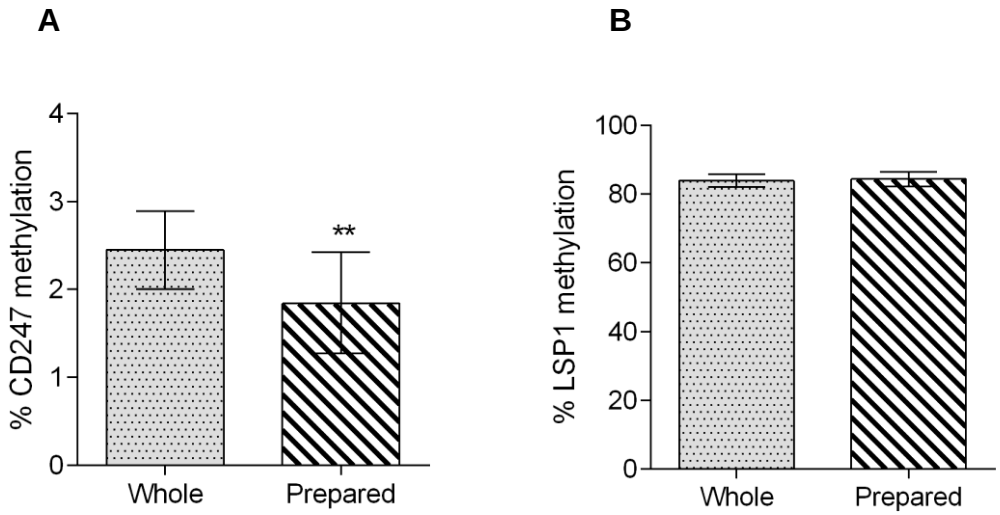


Figure 3.11 *CD247* and *LSP1* methylation in whole and prepared sperm

Methylation of the WBC markers *CD247* (n=16) (A) and *LSP1* (n=26) (B) in whole and prepared sperm. Independent t-tests were used to find significant differences between groups. Error bars represent upper and lower 95% CIs. ***p*<0.01.

3.2.3 DNA methylation and ART outcomes

To determine whether differential methylation was associated with altered fertilisation, cleavage and pregnancy rates, global and imprinted gene

methylation were compared between IVF and ICSI patients and correlated with outcomes in each group.

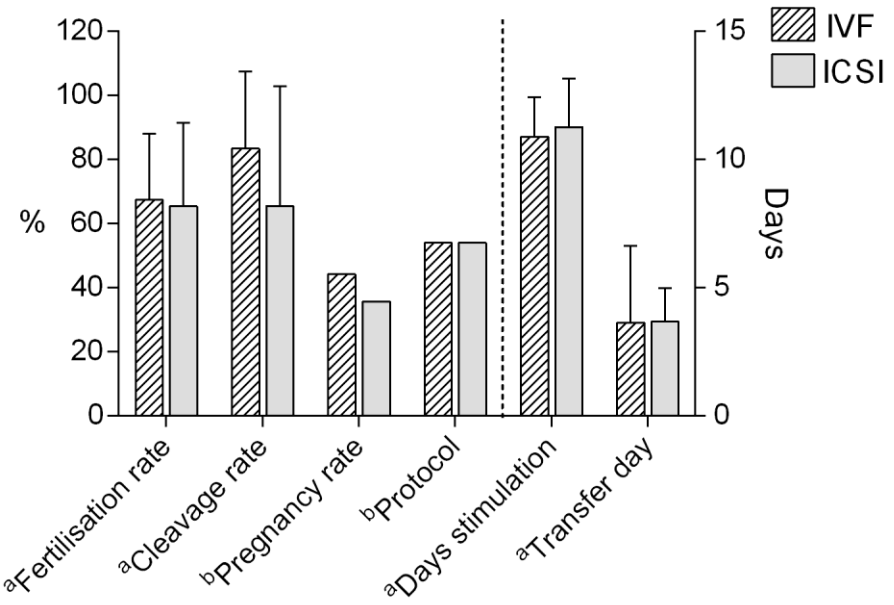


Fig. 3.12 IVF and ICSI data

There were no significant differences in treatment conditions or outcomes between IVF (n=52) and ICSI (n=37) groups as determined by the Mann-Whitney U test for non-parametric continuous variables ([a]) and χ^2 test for categorical variables ([b]). Data sets either side of the dotted line are plotted on the left and right y-axes. Error bars represent standard deviation values. Protocol represents %patients that received the LD 21 protocol.

3.2.3.1 Comparison of DNA methylation in IVF and ICSI patients

3.2.3.1.1. ART demographics and outcomes

ART data was collected for IVF and ICSI patients and results were interpreted separately for DNA methylation analysis. To identify differences between the two types of ART treatment, parameters including protocol type, days of stimulation, and transfer day, and outcomes including fertilisation, cleavage and pregnancy rates, were compared. There was no significant difference in age, % fertilisation, % cleavage or % pregnancy between IVF and ICSI groups. However, cleavage rates were considerably higher in the

IVF group than the ICSI group at 83.5% and 65.5%, respectively. Pregnancy rates were also higher in the IVF group compared with the ICSI group (44.2% and 35.7%, respectively), however this was not significant.

3.2.3.1.2 DNA methylation in IVF and ICSI patients

Sperm DNA methylation was compared between men undergoing IVF and those undergoing ICSI to determine whether differences in success rates were related to sperm methylation. The analysis showed that *SNRPN* and *DAZL* methylation were significantly higher in the ICSI group compared with the IVF group (p=0.01 and p=0.002, respectively) (fig. 3.13). None of the other genes nor global methylation showed significant differences between the groups.

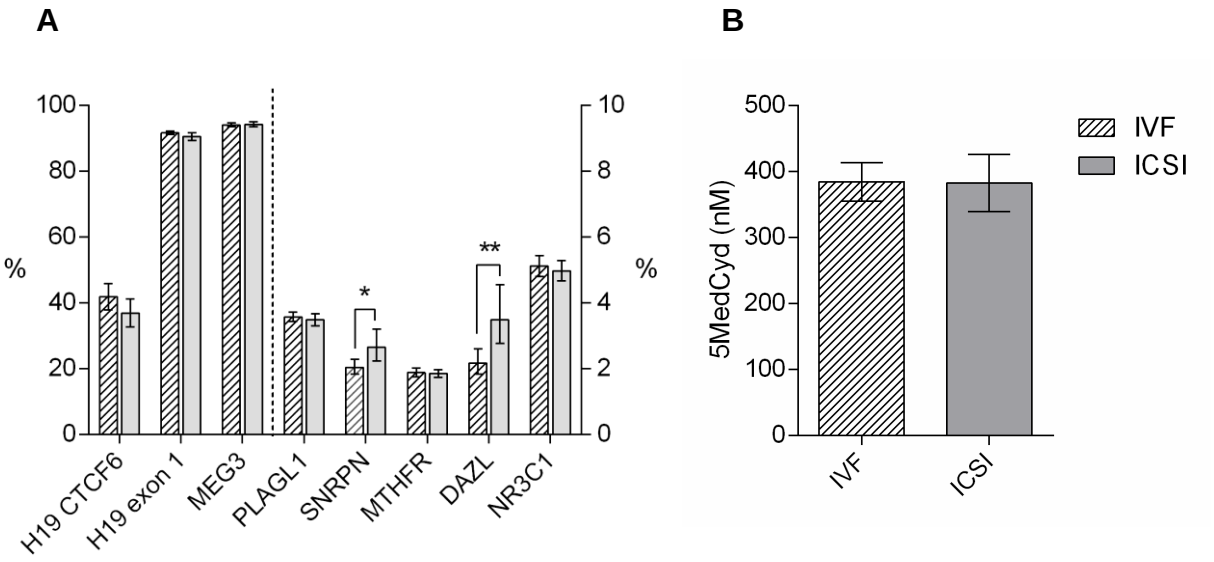


Figure 3.13 Sperm DNA methylation in IVF and ICSI patients

Gene-specific (A) and global DNA methylation (B) means and 95% CI are shown. Data sets either side of the dotted line (A) are plotted on the left and right y-axes. Independent t-tests were carried out to identify differences between the groups. Error bars represent upper and lower 95% CIs. *p<0.05, **p<0.01.

3.2.3.2 DNA methylation and ART outcomes

3.2.3.2.1 Univariate analysis of DNA methylation in whole sperm with ART outcomes

To identify whether aberrant sperm methylation of whole sperm may be linked to negative ART outcomes, methylation data was correlated with fertilisation and cleavage rates for IVF and ICSI patients. The results show that global DNA methylation is significantly negatively correlated with fertilisation rate in IVF patients (p=0.034) (fig. 3.14). No gene-specific methylation was found to be associated with fertilisation or cleavage rates. In IVF patients, *NR3C1* methylation was higher in those who successfully achieved pregnancy compared with those who did not (p=0.027) (appendix 13, table 5).

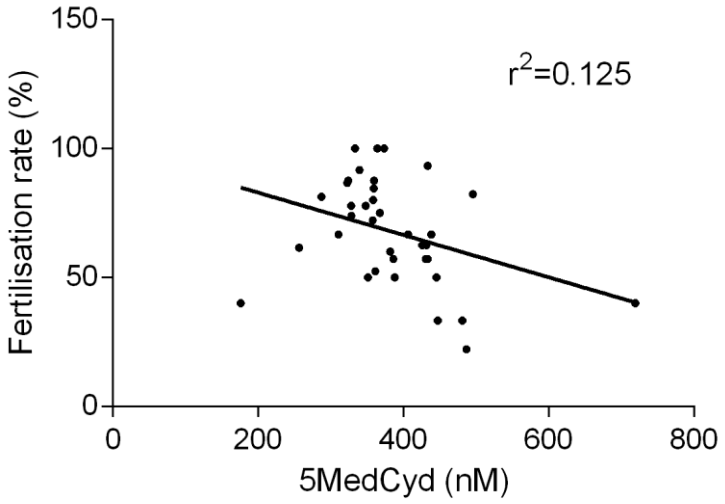


Fig. 3.14 Global DNA methylation and fertilisation rate

5MedCyd concentration was negatively correlated with fertilisation rates in IVF patients (n=36) (p=0.034).

3.2.3.2.2 Multivariate analysis of DNA methylation in neat semen and ART outcomes

To determine whether global methylation is truly linked to fertilisation rates, and whether NR3C1 methylation is linked to a greater chance of pregnancy, multiple linear regression and logistical regression were carried out, respectively. The regressions controlled for factors that primarily influence ART outcomes, including female age (which is known to be the major prognostic factor). Male age was also controlled for, despite not previously being associated with DNA methylation in this study (table 3.8), due to existing evidence that male age can be an influencing factor for ART outcomes. Protocol type and the duration of stimulation of the female partner for egg collection were included as independent control variables, as well as the transfer day for pregnancy outcomes. Results are shown in table 3.20.

Table 3.20 Multiple regression analysis of global methylation and fertilisation rates in IVF patients

Independent variable	Unstandardised B-coefficient	Standardised β-coefficient	Sig (p-value)	ANOVA (p-value)
5MedCyd (nM)	-0.071	-0.305	**0.055**	
Female age (years)	2.364	0.452	**0.011***	
Male age (years)	-1.199	-0.259	0.138	0.031
Days stimulation	0.646	0.055	0.731	
Protocol	0.719	0.018	0.907	

The association between global methylation and fertilisation rates in IVF patients was analysed using multiple linear regression while controlling for female and male age, duration of stimulation (days), and protocol type (LD 21 or antagonist). *p*-values indicate the significance of each independent variable on fertilisation rates while controlling for all other variables. Standardised β values represent the number of SD changes expected in the outcome variable for 1 SD change in the independent variable, while all other factors are held constant. Unstandardised B values are the coefficients in their original units.

Table 3.21 Logistical regression for *NR3C1* methylation and pregnancy outcome

	Sig. (*p*-value)	Odds ratio
NR3C1 %	0.092	0.000
Protocol	0.418	0.507
Female age	0.83	0.975
Male age	0.754	1.039
Days stimulation	0.801	1.076
Transfer day	0.161	0.601

Significance (*p*) values and odds ratios are shown for the likelihood of each of the IVs contributing to a change in the DV. There were no significant associations ($p>0.05$).

Table 3.22 Prepared sperm DNA methylation and ART outcomes

	5MedCyd (nM) (n=5)	H19 DMR % (n=16)	PLAGL1 % (n=16)	MTHFR % (n=15)	PEG10 % (n=16)
%fertilisation[a]					
r	-0.451	-0.23	0.02	-0.052	-0.319
p-value	0.446	0.391	0.933	0.855	0.229
%cleavage[a]					
r	0.058	0.31	-0.184	0.148	-0.146
p-value	0.926	0.243	0.495	0.599	0.589
%pregnancy[b]					
t	-	0.83	0.038	0.69	1.965
p-value	-	0.42	0.971	0.503	0.07

Statistical analysis was performed using the Pearson's correlation for continuous variables ([a]) and the independent t-test for categorical variables ([b]).

The linear regression analysis showed that global methylation was no longer

statistically significantly associated with fertilisation rates in IVF patients. The regression showed that the main determining factor for fertilisation rates was female age (p=0.011), while male age, duration of stimulation and protocol type did not significantly contribute.

Logistical regression for the association between *NR3C1* methylation and pregnancy likelihood did not show significance for any of the independent variables (table 3.21).

3.2.3.2.3 DNA methylation in prepared sperm and ART outcomes

As it is the prepared sperm which is used for IVF and ICSI, the association between methylation of these cells and ART outcomes was investigated. Pearson's correlation between prepared sperm methylation and fertilisation rate and cleavage rate showed no significant associations (table 3.22). In addition, methylation levels were not different in prepared sperm from those who achieved pregnancy and those who did not.

3.3 Discussion

This study set out to determine the influences of health and lifestyle factors and environmental exposures on sperm DNA methylation, and whether disrupted methylation is associated with sperm parameters and IVF and ICSI outcomes. DNA methylation at CpG sites plays a critical role in genome function, including establishing parent-of-origin imprints in sperm and oocytes. Due to the importance of imprinted genes in embryonic development and to existing research that shows that aberrant methylation at these sites is found in infertile men, the genes *H19, MEG3, PLAGL1* and *SNRPN* were investigated. The *MTHFR* and *DAZL* genes are non-imprinted genes which have roles in the folate pathway and in spermatogenesis, respectively. As DNA methylation is one of the primary regulators of gene expression, aberrant methylation of these genes could have consequences for sperm function. The *NR3C1* gene is involved in the stress response pathway and has been widely shown to undergo methylation changes in response to stress and environmental pressure; it was therefore included in this study as a potential marker for stress. Finally, global methyl-cytosine levels were measured as a wider marker for methylation changes, including methylation at non-CpG sites and in non-regulatory regions such as repetitive elements. The main purpose of this study was therefore to determine which lifestyle and environmental factors influence global and gene-specific methylation and to identify whether this is consequential for semen parameters and ART outcomes.

The major finding of the association between lifestyle and environment and sperm DNA methylation, was that global methylation levels were significantly higher in men who were exposed to glues, adhesives or resins. This contradicts existing research that suggests that exposure to damaging environmental toxicants cause a decrease in global sperm methylation (Miao *et al.,* 2014). Global methylation was also higher in men who were meat and fish eaters compared with meat eaters alone, however the number of

reported meat-only eaters was also very low (n=5) and was not investigated further.

Factors that are thought to pose the greatest risk for male infertility are age, BMI and smoking (Begueria *et al.,* 2014; Eisenberg *et al.,* 2013; Hamad *et al.,* 2014). However, this study did not find any association between these health risks and global or gene-specific methylation. However, *SNRPN* methylation was found to be higher in ex-smokers than in those who had never smoked, but this significance was lost when controlling for other factors. *SNRPN* methylation was also not correlated with the number of years that the individual had smoked. Jenkins *et al.* (2014) found an overall increase in global methylation levels in sperm while simultaneously showing site-specific hypomethylation of genes. In this study, a comparison of global methylation levels with gene-specific methylation did not show any significant correlations.

The association between DNA methylation and sperm parameters showed some strong correlations. *DAZL, MEG3* and global methylation were significantly negatively associated with sperm concentration and sperm motility. When looking at the study population as a whole, sperm concentration was found to be highly negatively correlated with methylation of the *DAZL* gene promoter. This strong association was still present when subjects were separated into normozoospermic and oligozoospermic groups, in which case methylation was significantly higher in the latter. As DNA methylation at regulatory sites in genes generally leads to gene silencing, this provides evidence that hypomethylation of the *DAZL* promoter as an essential regulatory feature for expression of this gene. Moreover, *DAZL* methylation was correlated with sperm concentration in men who had concentration in the normal range ($\geq 15 \times 10^6$/ml), indicating that inhibition of spermatogenesis occurs in a dose-dependent manner based on *DAZL* methylation. This is in concordance with a study by Navarro-Costa *et al.* (2010) who found that the CpG island spanning the *DAZL* promoter had a higher number of methylated sites in sperm from oligoasthenozoospermic men compared to normozoospermic men. The method of analysis in the

study referred to was bisulphite genomic sequencing of cloned fragments of sperm DNA. The methylation status of CpG sites is then shown as either unmethylated, hemi-methylated or methylated, and the % methylation quantified from the number of methylated clones. In contrast, the present study used bisulphite pyrosequencing, for which the output results in a read of the percentage of methylated DNA copies (i.e. cells) in a sample, and is considered to be more robust. The analysis showed that there was a large variation in *DAZL* methylation within the population, with some samples having methylation of over 20%, which would have a considerable impact on expression levels. CpG site-specific analysis also revealed that all sites within the *DAZL* promoter region were correlated with the mean methylation, therefore the aberrant methylation we have found associated with low sperm concentration is broadly affecting all CpG sites rather than a select few. Li *et al.* (2013) showed similar results for the effects of *DAZL* on semen parameters and also found that the 6th CTCF binding site of *H19* was hypomethylated in oligozoospermic men, a trend which was not found here. The only exposure factor that had an effect on *DAZL* methylation was exposure to metal dust or fumes, however this was not significant. Metals, such as lead and cadmium, are considered to be damaging to male reproductive function (Wirth and Mijal, 2010), and could be one of the mechanisms in which elevated *DAZL* methylation leads to lower sperm concentration, however further research is needed to clarify this.

The *MEG3* IG-DMR was the only imprinted gene region to show a significant correlation with semen parameters. Interestingly, *MEG3* methylation was negatively correlated with % progressive sperm motility which is contrast to two studies which showed a negative influence of *MEG3* hypomethylation: Kobayashi *et al.* (2007) found that *MEG3* methylation was lower in oligozoospermic men, and El Hajj *et al.* (2011) found lower *MEG3* methylation in infertile men compared to fertile men, but did not describe any effects on sperm parameters. Furthermore, *MEG3* was not linked to any lifestyle or environmental exposures in this study.

Global DNA methylation was significantly positively correlated with % immotile sperm following multivariate analysis. This is in accordance with studies which found that methylation of *LINE-1,* a surrogate marker for global methylation, was associated with lower sperm motility (Tian *et al.,* 2014) and this suggests that hypermethylation of sperm DNA is linked to an increase in % immotile sperm. However, this directly contrasts the findings of Montjean *et al.* (2015) who found that global methylation was positively correlated with sperm progressive motility and sperm concentration, and used a similar ELISA method to that used in this study. The study also found an inverse relationship between global methylation and DNA fragmentation, suggesting that low levels of global methylation signify a general decrease in genomic integrity. Critically, a recent study which analysed DNA damage in the same cohort of men (Altakroni, 2015) found that high levels of DNA double strand breaks were found in sperm samples with high numbers of % immotile sperm. One of the sources of such damage is oxidative stress, and ROS levels have also been linked to a decrease in global methylation in sperm (Tunc and Tremellen, 2009). Given this evidence, lower methylation might be expected to be characteristic of samples with a high number of immotile sperm, however this is not the case according to the results of this study. In accordance with the finding that global methylation is positively correlated with % immotile sperm, the present study also showed a negative correlation with fertilisation rate in IVF patients, although insignificant. This again contradicts existing evidence that points to hypomethylation as a cause of poor ART outcomes (Benchaib *et al.,* 2005). Apart from global methylation, the major determinant of fertilisation outcomes was maternal age, which is already widely acknowledged.

A comparison of DNA methylation between IVF and ICSI patients revealed that *DAZL* methylation was significantly higher in the sperm of men undergoing ICSI treatment, which likely reflects the fact that ICSI treatment is most often used for men with poor sperm quality. There was no significant trend in *DAZL* methylation between fertile and infertile men, however, which can be explained by male infertility caused by factors other than low

concentration, such as poor motility which was not correlated with *DAZL* methylation. Additionally, infertile men can show normal semen parameters. Therefore the primary function of *DAZL* appears to be in sperm production, but as the results show, it has no effect on ART outcomes.

Interestingly, the *MTHFR* gene showed higher methylation levels in infertile men yet did not show any relation to semen parameters. *Mthfr* plays an essential role in the folate/homocysteine pathway that produces methyl donors for the methylation process. Wu *et al.* (2010) found that *MTHFR* promoter hypermethylation was associated with idiopathic male infertility and, importantly, that men with normal semen parameters had higher methylation than fertile men. This supports the results of this study, which suggest that increased *MTHFR* methylation is linked to infertility without causing differences in standard semen parameters. This study also examined associations between *MTHFR* methylation and other genes to determine whether aberrant *MTHFR* expression might affect methylation at other sites, as has been shown elsewhere (Rotondo *et al.,* 2013), however no significant trends were found. This may be due to the low number of CpG sites analysed compared to the studies above, which despite being located in the same CpG island spanning the *MTHFR* promoter, may have missed more informative CpG sites. Considering the indispensable roles of DNA methylation in embryogenesis, it may be expected to see some correlations between *MTHFR* gene methylation and ART outcomes. Indeed, *MTHFR* hypermethylation has previously been correlated with spontaneous miscarriage (Rotondo *et al.,* 2012); however no significant associations were found for fertilisation, cleavage or pregnancy rates in this study.

SNRPN showed a similar trend to *DAZL* in that it showed higher methylation in the sperm of men undergoing ICSI compared to men undergoing IVF, yet this gene was not correlated with sperm parameters nor was it significantly different when comparing fertile and infertile men. This maternally imprinted gene typically has low levels of methylation in sperm, compared with oocytes in which *SNRPN* methylation is high (Geuns *et al.,* 2003). Again, as sperm used for ICSI is generally of lower quality, this significant increase in

methylation may be associated with substandard sperm. Indeed, *SNRPN* hypermethylation has previously been linked to low sperm motility (Botezatu *et al.,* 2014), which is the major determining factor for whether IVF or ICSI treatment is carried out. *DAZL* and *SNPRN* were in fact highly positively correlated with each other, and may be acting synchronously to affect sperm.

Differences in methylation between neat semen and prepared sperm were analysed as prepared sperm is used for IVF and ICSI, and therefore the integrity of this DNA is important for embryogenesis. This study found that prepared sperm had consistently significantly higher levels of methylation than sperm from neat semen. This included global methylation, the paternally imprinted *H19* exon 1 and *PLAGL1,* the maternally imprinted gene *PEG10* and *MTHFR.* Whether this increase in methylation is a positive or negative change is an important question. As already known, an increase in methylation of the hypomethylated genes *PEG10* and *MTHFR* could be detrimental as it reduces expression levels. However, an increase in global methylation is thought to be beneficial as it indicates good genomic stability (Rodriguez *et al.,* 2006). In the sister study on the same male cohort (Altakroni, 2015), the level of DNA strand breaks was lower in prepared sperm, suggesting the DNA of the purified fraction is of higher quality, which supports evidence from published studies (Jayaraman *et al.,* 2012). Given this evidence, it can be concluded that the prepared sperm fraction had higher levels of methylation, which is indicative of selection of good quality sperm with high quality DNA. Methylation of prepared sperm was not associated with fertilisation, cleavage and pregnancy rates. Although this is in conjunction with other studies which found no association between prepared sperm methylation and ART outcomes (Camprubi *et al.,* 2012), a major limitation here was the low number of prepared samples for analysis. Therefore, a definite conclusion cannot be drawn on the effects of prepared sperm methylation on ART outcomes.

The use of a leukocyte-expressing gene was investigated as a prognostic marker for WBC contamination in sperm because specific methylation fingerprints can be observed for different cell types. This was investigated

here based on the idea that WBC genes are heavily methylated and silenced in sperm but unmethylated and expressed in contaminating WBCs. Firstly, the *CD247* gene was investigated as it forms part of the T-cell receptor and is therefore unmethylated in these cells. However, contrary to expectations, this gene also showed low levels of methylation in whole and prepared sperm, which may suggest some role for it in sperm function. Furthermore, we would expect a WBC gene such as this to show higher levels of methylation in prepared sperm, however the opposite trend was observed. Again, this is reasonable if *CD247* has some role in sperm function, and is therefore unmethylated in fractionated high quality motile sperm. Secondly, the *LSP1* gene was investigated as a possible marker of WBC contamination. This gene showed high levels of methylation in sperm as expected, indicative of gene silencing. However there was no difference in methylation between prepared sperm and neat semen. This lack of finding may be due to semen samples containing relatively low WBCs. WBC counts were not available for this study, but would be required to confirm the differences in methylation between neat and prepared sperm and between different semen samples. This method for detection of different cell types by methylation fingerprinting has been used successfully in blood and tumour tissue samples (Wieczorek *et al.,* 2009; Sehouli *et al.,* 2011), however no study has yet used this method to quantify non-sperm cell DNA in neat semen. Although determination of WBC levels in this manner would have little relevance in the clinical setting as staining methods for WBC determination are straight forward and low-cost, this would be a useful and important control for sperm sequencing studies.

3.3.1 Conclusion

This study found that the contribution of lifestyle and environmental factors to changes in DNA methylation of imprinted and non-imprinted genes was low, but may be more significant for global DNA methylation. Although little association was found between imprinted gene methylation and sperm quality, aberrant methylation of a spermatogenesis-associated gene, *DAZL*,

is directly linked to lower sperm concentration, but has no impact on ART outcomes. Furthermore, global DNA methylation may be an important marker of overall sperm quality and ART outcomes, as global methylation was found to be associated with sperm motility and fertilisation rates in IVF patients. Overall, the results suggest that aberrant sperm methylation may have consequences for sperm function and ART outcomes.

Chapter 4

Effects of lifestyle and environment on semen parameters and ART outcomes

4.1 Introduction

Several studies have reported a decrease in semen quality, such as the reductions in semen volume, reductions in sperm concentration, motility and normal morphology (Carlsen *et al.,* 1992; Auger *et al.,* 1995; Rolland *et al.,* 2013; Romero-Otero *et al.,* 2015; Centola *et al.,* 2016). Infertility is now experienced by 1 in 6 couples, with 30% of cases explained by factors in the male partner (European Society of Human Reproduction and Embryology, 2014). Therefore, determining the main effectors of semen parameter changes is paramount. These decreasing fertility trends have paralleled lifestyle changes in Western countries such as an increase in sedentary behaviour and a transition to diets high in sugar, saturated fat and salt (Department of Health, 2010; Public Health England 2014), calling into question the contribution of environmental and lifestyle factors to male infertility.

Diet has been shown to be an important factor associated with good fertility outcomes and consumption of certain foods has been linked with lower sperm quality (Afeiche *et al.,* 2013; Gaskins *et al.,* 2012; Chiu *et al.,* 2014). Furthermore, a lack of physical activity has also been shown to have detrimental effects on fertility (Gaskins *et al*., 2015). Another common and detrimental characteristic of modern society is occupational stress (Labour Force Survey, 2015). Stress has been shown to interfere with fertility treatment (reviewed in Campagne*,* 2006) and direct evidence of the damaging effects of stress on sperm has been shown previously (Janevic *et al.,* 2014). Exposure to environmental toxicants is also a risk factor for

infertility: consumption of pesticide-contaminated foods has been linked to lower numbers of morphologically normal sperm (Chiu *et al.,* 2015) and occupational exposures such as glyxol ethers have been linked to a reduction in motile sperm count (Cherry *et al.,* 2014). It is possible that a combination of some of these modifiable lifestyle factors lead to substandard semen quality. Additionally, advancing age has been shown to be an important risk factor for poor semen quality (Johnson *et al.,* 2015) which is relevant at this time as more couples choose to delay child-bearing by some years (Royal College of Obstetricians and Gynaecologists, 2009).

As the number of couples undergoing ART worldwide continues to rise, improving success rates is a priority. It is widely known that maternal factors, particularly age, have a major influence on both natural pregnancy and ART outcomes (Jolly *et al.,* 2000; Sazonova *et al.,* 2011). However, paternal influences do play an essential role. Sperm quality can affect fertilisation, cleavage rates, and embryo quality indicating that sperm can affect pre-implantation development (Loutradi *et al.,* 2006), however the influence on pregnancy rates is not conclusive (van der Westerlaken *et al.,* 2009). It is possible for lifestyle and environmental factors to affect ART outcomes without causing clear changes to standard semen parameters, instead causing alterations at the molecular or genetic level (Anifandis *et al.,* 2013; Wegner *et al.,* 2010). In this case, the contribution of paternal lifestyle effects to ART outcomes could be significant.

4.1.1 Aims

The aims of this chapter were to

1. To identify which lifestyle and environmental factors associate with sperm concentration, motility, total sperm count and semen volume in a population of men attending a fertility clinic.
2. To determine whether sperm quality in neat semen and in prepared sperm is related to fertilisation, cleavage and pregnancy rates in IVF and ICSI patients.

3. To determine whether paternal lifestyle factors are associated with fertilisation, cleavage and pregnancy rates in IVF and ICSI patients.

4.2 Results

4.2.1 Lifestyle and semen parameters

4.2.1.1 Semen parameters of the study population

4.2.1.1.1 Semen parameters raw data

A total of 169 participants were included in this study. Nine patients presenting with a current varicocele were excluded from the study, bringing the total to 160 men. Two participants did not complete the questionnaire. Table 4.1 shows the original semen parameters data.

Table 4.1 Semen parameters of the study population

Variable	n	Mean(SD)	Median	25th percentile	75th percentile	Min	Max
Concentration (x10^6/ml)	157	60.3 (57.5)	43.0	23.0	79.75	0.5	362.0
Progressive motility (%)	157	48.29(16.76)	50.0	39.0	60.0	0.0	83.0
Immotile cells (%)	157	42.49(15.63)	40	33.0	51.5	9.0	96
Volume (ml)	155	3.14(1.43)	3.0	2.2	3.8	0.0	8.1
Count (x10^6)	155	181.75(169.99)	130	55.2	257.4	0.0	840.0

4.2.1.1.2 Normalisation of semen parameters

For parametric statistical analysis purposes, continuous data must show a Gaussian, or normal bell-shaped, distribution, therefore the frequency distributions of sperm concentration, progressive motility, immotile cells,

volume and sperm count were analysed on histograms and Q-Q plots. Sperm concentration and sperm count showed non-normal data and were mathematically transformed to $\log_{10}$ (see section 2.1.2 and appendix 6). Geometric means and back-converted 95% CI are therefore presented in the results for these parameters. % progressively motile, % immotile sperm and semen volume are presented as raw data values.

4.2.1.1.3 Semen parameters correlation matrix

Poor quality semen often manifests as abnormalities in more than one sperm parameter. The association between sperm concentration, % PM, % immotile, volume and total sperm count were therefore analysed to determine whether these parameters are linked. Pearson's correlation showed that sperm concentration was strongly positively correlated with % PM sperm and negatively correlated with % immotile sperm (table 4.2). % PM and % immotile cells were highly negatively correlated which is expected as these variables are oppositely linked. Sperm count was highly correlated with concentration and volume, which was expected as total count is determined directly by these factors. Sperm count was also highly positively and negatively correlated with %PM and %immotile, respectively. Finally, seminal volume was not correlated with parameters other than sperm count. Seminal volume is highly variable, and as sperm count is directly linked to this, it must be taken into account that changes in sperm count could be as a result of natural variation in volume e.g. seasonal variation (Di Georgi *et al.,* 2015), rather than lifestyle-induced changes. Volume can also be reduced by male reproductive tract infections which can affect accessory gland function, including the prostate and seminal vesicles, which secrete seminal fluid and its components (Marconi *et al.,* 2009).

Table 4.2 Semen parameters correlation matrix

	PM (%) n=157	Immotile (%) n=157	Volume (ml) n=155	Count (x10^6) n=157
Concentration (x10^6/ml)				
r-statistic	**0.348****	**-0.269****	-0.05	**0.84****
p-value	**0.000**	**0.001**	0.946	**0.000**
Progressively motile (%)	--			
r-statistic		**-0.92****	0.091	**0.312****
p-value		**0.000**	0.262	**0.000**
Immotile (%)	--	--		
r-statistic			-0.082	**-0.245****
p-value			0.312	**0.002**
Volume (ml)	--	--	--	
r-statistic				**0.345****
p-value				**0.000**

Pearson's correlation, r, and sig. (*p*) values are shown for associations between semen parameters.

4.2.1.1.4 Abstinence, seminal volume and sperm count

The duration of abstinence is one of the biggest predictors of seminal volume; therefore to determine whether volume, and consequently sperm count, was affected by abstinence time, differences were assessed between the number of days the participants abstained for (fig. 4.1). Most participants reported to have abstained from intercourse or masturbation for 2-5 days (n=122), while some had abstained for 6 days (n=4), 7 days (n=1), 8 days (n=1), 9 days (n=1) and 10 days (n=1). Twenty-seven participants did not give an answer to this question in the questionnaire. Mean semen volume showed an overall decrease between those abstaining for 2 days and those abstaining for 6 days, however the only significant correlation was between days 3 and 4 which showed an increase (p=0.037). Days 3 and 4 had the highest numbers of participants abstaining (n=53 and n=44) which suggests this is the most likely representation of true population. Sperm count was not significantly different between any of the time points (p=0.059).

4.2.1.2 Study population health and lifestyle demographics

4.2.1.2.1 Health and lifestyle 3 months prior to the study

In this study, we were interested in modifiable and non-modifiable risk factors that may be associated with aberrant sperm parameters such as age and illness. Therefore we initially analysed the data set as a whole to look at the overall demographics and lifestyle trends of the participants.

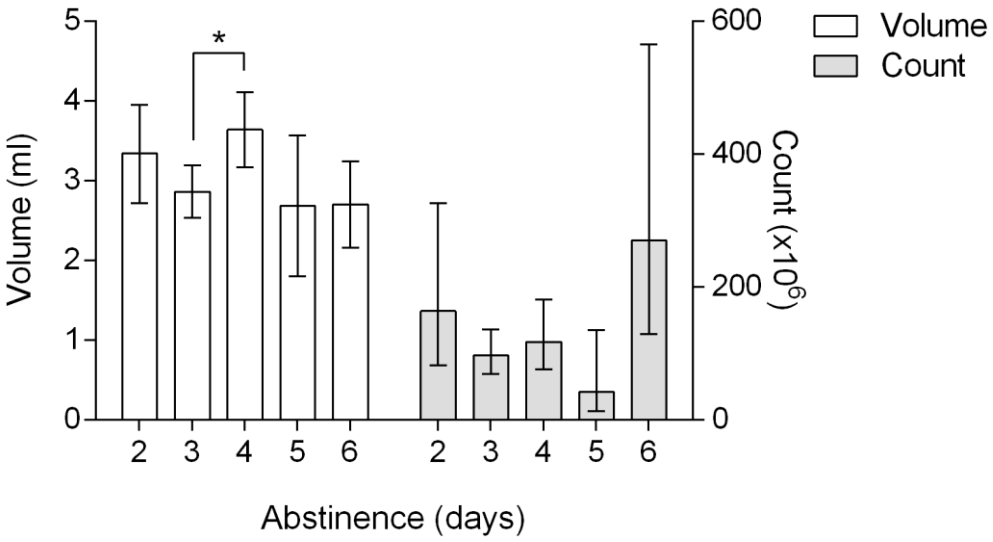


Figure 4.1 Effects of abstinence time on semen volume and sperm count

There was no clear trend in semen volume and sperm count with duration of abstinence. One-way ANOVA statistics and Tukey post-hoc tests identified a significant difference in volume between three and four days. Significant differences are flagged (*). Error bars represent 95% CI. For sperm count, the mean represents the geometric mean. Numbers of men abstaining for 2, 3, 4, 5 and 6 days were 12, 53, 44, 13 and 4, respectively.

Participants' ages (years) were collected by the hospital as part of the patient consultation. The mean age of the study population was 35.86 years (23 to 57 years) (table 4.3). BMI was categorised as healthy, <25 kg/m^2; overweight, 25-29.9 kg/m^2; and obese ≥30 kg/m^2 and these made up 49%, 31.7% and 13.9% of the study population, respectively. There was also one

underweight case (17.5 kg/m^2). 50% of men were classed as physically active and 50% were insufficiently active according to their leisure score index (LSI) which was calculated from the amount of moderate and strenuous exercise they reported (table 4.3). Alcohol consumption was moderate with 69% of men consuming at least one drink per week (table 4.4) and within the drinking group the 75th percentile was 19.15 units, which is under the recommended limit of 21 units per week (table 4.3). The mean and median were similar indicating that most men were light or moderate drinkers. 10.3% of men said they did not wear boxer shorts. The number non-smokers (56.7%) was roughly equal to that of ex-smokers (43.3%) with a wide variation in the number of years smoked (0.5-33 years).

With regards to health, 17.2% of men reported to have suffered from fever or flu in the 3 months prior to the study. 14% of men reported suffering from a work-related illness and 9.6% of men said that a work-related illness was due to stress (table 4.4). 43.7% of men were ex-smokers and within this group, the mean duration of smoking was 12.76±6.89 years.

Table 4.3 Lifestyle demographics on a continuous

Variable	Mean (SD)	n	Median	25th percentile	75th percentile	Min	Max
Age (years)	35.8 (5.52)	157	35.0	32.0	39.0	23.0	57.0
BMI (kg/m^2)	25.93 (3.73)	151	25.3	23.2	28.1	17.5	37.2
Alcohol (units/week)	14.16 (9.15)	109	13.0	7.8	19.15	2.0	52.0
Leisure score index	27.83 (29.89)	156	23.5	9.0	36.0	0.0	250.0
Smoking (years)	12.83 (6.92)	68	12.5	7.25	18.0	0.5	33

Descriptive statistics are shown. Data for alcohol consumption and smoking duration is taken from only those who reported to drink alcohol more than once per week and who were ex-smokers.

Table 4.4 Participants health and lifestyle 3 months prior to the study

Variable	Number (%)
Fever or flu	
No	130 (82.8)
Yes	27 (17.2)
Job illness	
No	135 (86.0)
Yes	22 (14.0)
Illness due to stress	
No	142 (90.4)
Yes	15 (9.6)
Smoking history	
Non-smoker	89 (56.7)
Ex-smoker	68 (43.3)
Alcohol consumption	
<1 drink per week	48 (30.5)
>1 drink per week	109 (69.05)
Underwear	
Boxer shorts not worn	16 (10.3)
Boxer shorts worn	139 (89.7)
Leisure score index	
<24 points	78 (50.0)
≥24 points	78 (50.0)
BMI (kg/m^2)	
<25	74 (49.0)
25-29.9	56 (37.1)
≥30	21 (13.9)

4.2.1.2.2 Chemical and physical exposures

Men were then asked about their exposure to certain chemical and physical agents in the 3 months prior to the study. The number of men exposed to each factor was analysed to determine which factors could be investigated for their effects on semen parameter quality, i.e. which factors had sufficient numbers of men exposed and not exposed. Table 4.5 shows the frequencies of reported exposures to each factor. It shows that less than 10% of participants were exposed to pesticides, herbicides, fertilisers, dry cleaning fluids, PVC or plasticisers, extremely hot environments, non-ionizing radiation, X-rays or reported driving for long periods. These variables were not included in any further analysis.

Table 4.5 Chemical and physical exposures 3 months prior to the study

Exposure	Number (no/yes)	% Yes
Metal dust or fumes	129/29	18.4
Pesticides	156/2	1.3
Herbicides	143/10	6.3
Fertilisers	141/11	7.0
Oils or greases	107/47	29.7
Detergents and soaps (at job only)	94/60	38.0
Glues, adhesives or resins	109/44	27.8
Paints, varnishes or lacquers	111/42	26.6
Printing inks or dyestuffs	134/19	12.0
Dry cleaning fluids	152/1	0.6
PVC or plasticisers (at job only)	144/9	5.7
Other solvents	114/38	24.1
Extremely hot environments	140/13	8.2
Heavy vibrating machinery, equipment or vehicles	133/20	12.7
Nonionizing radiation	142/11	7.0
Driving for long periods	145/8	5.1
X-rays	149/4	2.5

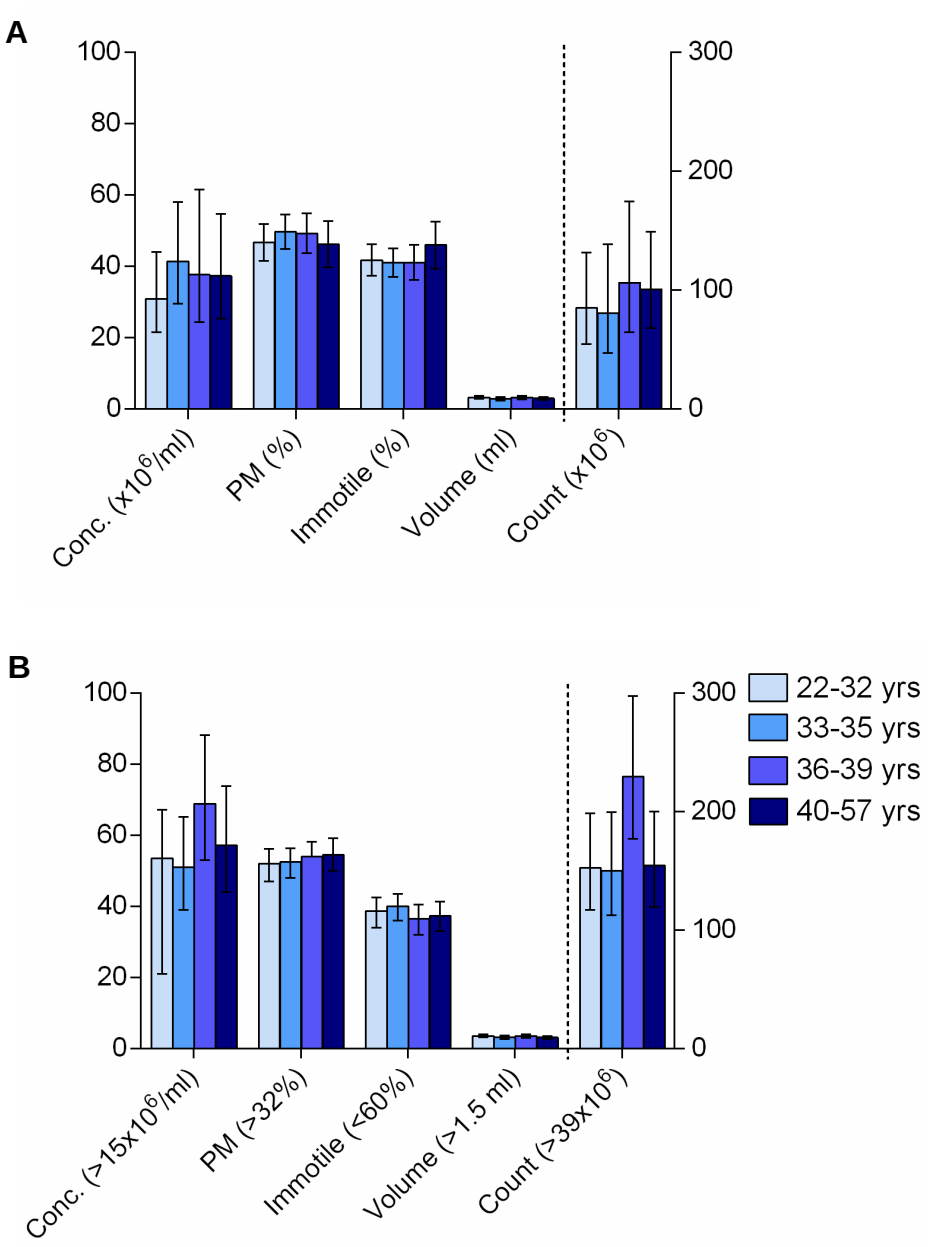


Figure 4.2 Effects of age on semen parameters

Analysis of semen parameters and male age in the entire data set (A) and in men with normal semen parameter values (B). Age was categorised into quartiles (key) and a one-way ANOVA analysis of differences in each parameter between quartiles was carried out. No significant differences were found in semen parameters with age. Error bars represent upper and lower 95% CI.

4.2.1.3 Semen parameters, health and lifestyle

4.2.1.3.1 Semen parameters and age

To investigate the association between age and semen parameters, age (years) was analysed on a continuous scale and also separated into categorical quartiles. Age was not correlated with sperm concentration, % progressively motile cells, % immotile cells, seminal volume or total sperm count on a continuous or categorical scale (fig. 4.2 A). As the severity of abnormalities in men with sperm parameters below the normal reference limits may hide the effect of age, semen parameters were also analysed when restricted to the WHO recommendations for normal values: concentration, $\geq 15 \times 10^6$ cells/ml; progressive motility, ≥32%; immotile cells <60%, total sperm count, $\geq 39 \times 10^6$ cells and seminal volume, ≥1.5 ml. Men with normal semen parameters also showed no associations with age (fig. 4.2 B).

Table 4.6 Semen parameters and exposures in the 24 hours prior to the study

	n	Concentration (x10^6/ml)	%PM	%immotile	n	Volume
Smoke exposure						
Not exposed	143	37.65(30.99-45.68)	47.69(44.78-50.59)	42.44(39.79-45.09)	141	3.12(2.88-3.36)
Exposed	14	25.78(11.61-55.87)	50.5(43.08-57.92)	42.43(35.58-49.27)	14	3.29(2.51-4.06)
t-statistic (p-value)		*1.132(0.259)*	*-0.584(0.56)*	*-0.003(0.998)*		*-0.42(0.675)*
Work attendance						
No attendance	51	37.33(26.17-53.06)	45.41(40.41-50.41)	44.84(40.25-49.44)	51	2.95(2.53-3.37)
Attendance	105	36.62(29.25-45.78)	49.19(45.91-52.47)	41.39(38.43-44.35)	103	3.24(2.97-3.51)
t-statistic (p-value)		*0.095(0.925)*	*-1.287(0.2)*	*1.293(0.198)*		*-1.187(0.237)*
Red meat consumption						
No	62	41.5(31.39-54.77)	48.9(44.65-53.15)	42.23(38.25-46.2)	60	2.2(2.67-3.33)
Yes	95	33.07(25.64-42.57)	47.27(43.71-50.84)	41.8(38.61-44.99)	95	3.24(2.93-3.55)
t-statistic (p-value)		*1.172(0.243)*	*0.58(0.563)*	*-0.146(0.884)*		*-1.035(0.302)*
White meat consumption						
No	97	33.75(26.2-43.42)	47.76(43.84-51.68)	42.86(39.34-46.37)	96	3.18(2.88-3.47)
Yes	60	40.46(30.58-53.48)	48.17(44.87-51.46)	41.8(38.61-44.99)	59	3.1(2.73-3.47)
t-statistic (p-value)		*-0.927(0.355)*	*-0.143(0.887)*	*0.443(0.658)*		*0.314(0.754)*
Fish consumption						
No	118	34.87(27.57-44.04)	49.59(46.52-52.66)	40.69(38.01-43.38)	116	3.22(2.95-3.5)
Yes	39	40.43(30.7-53.16)	42.85(37.21-48.48)	47.77(42.15-53.39)	39	2.93(2.54-3.32)
t-statistic (p-value)		*-0.822(0.413)*	***2.153(0.033)****	***-2.487(0.014)****		*1.111(0.268)*

Independent t-test statistics and significance (*p*) values are shown. Significant associations are highlighted in bold.
*$p<0.05$.

4.2.1.3.1 Exposures in the 24 hours prior to the study: effect on semen parameters

Sperm concentration was not correlated with any of the 24 hour exposure variables (table 4.6). This was the case when all samples were analysed and when outliers were excluded (>3x10^6/ml), and when an analysis was carried out only in men with normal sperm concentration (≥15x10^6/ml). Men who ate fish in the 24 hours before the study had lower % progressively motile sperm (p=0.033) and higher % immotile sperm (p=0.014). This was still significant when the number of progressively motile sperm was restricted to >10% (p=0.025) and when the number of immotile cells was restricted to <80%. However, neither of these trends were significant in sperm which had a minimum % PM of 32% (p=0.05). When samples with % PM of 32% were analysed, white meat consumption was linked to a reduction in % PM (p=0.018). Seminal volume was not correlated with any 24 hour exposures and this remained the case when extreme outliers were excluded, or when a samples ≥1.5 ml were analysed.

4.2.1.3.2 Semen parameters, health and lifestyle 3 months previous

An analysis of health and lifestyle in the 3 months prior to the study (table 4.7) found that semen parameters were not related to whether the participant had fever or flu in the previous 3 months, whether they had a job-related illness, or whether this illness was due to stress. Semen parameters were also not related to whether the participant was an ex-smoker or had never smoked, or whether they were a meat-eater or meat- and fish-eater. Participants who were physically active (LSI≥24) compared with those who were insufficiently active (LSI≤23) did not show differences in their semen parameters (table 4.7) nor was there a difference in semen parameters when looking at LSI on a continuous scale (table 4.8).

A decrease in % immotile sperm was found in men who wore boxer shorts (p=0.025), and this correlation was still significant in men who had normal motility parameters of ≥32% progressively motile cells but not when %

immotile cells was restricted to <80%. There were no significant differences in seminal volume with any of the lifestyle variables.

Alcohol consumption was measured as the number of units consumed per week and men were categorised as non- or light-drinkers (<1 unit per week), moderate drinkers (1-21 units per week) and heavy drinkers (>21 units per week). Sperm concentration was found to be significantly higher in the heavy drinking group than the moderate group (p=0.016). When only men with sperm concentration ≥15x10^6/ml were analysed, the trend was still significant (p=0.028). When analysed on a continuous scale (table 4.8), alcohol consumption was positively correlated with sperm concentration (p=0.04), however this was not significant when looking at samples with ≥15x10^6 cells/ml (p=0.101, n=128). Alcohol consumption was not associated with any other semen parameters.

Table 4.7 Semen parameters, health and lifestyle 3 months prior to the study

Variable	n	Concentration (x10^6/ml)	%PM	%immotile	n	Volume (ml)
Fever or flu						
No	130	36.87(30.19-44.99)	47.54(44.56-50.52)	42.95(40.2-45.71)	129	3.04(2.84-3.24)
Yes	27	33.76(19.66-57.49)	50.19(43.56-56.81)	39.78(34.33-45.22)	26	2.66(2.16-3.16)
t-statistic (p-value)		*0.351(0.726)*	*-0.729(0.467)*	*0.962(0.337)*		*1.489(0.139)*
Job illness						
No	135	36.82(30.04-45.08)	47.64(44.68-50.59)	42.83(40.12-45.54)	133	3.01(2.81-3.22)
Yes	22	33.41(20.04-55.29)	50.18(43.33-57.03)	39.82(34.0-45.64)	22	2.75(2.23-3.26)
t-statistic (p-value)		*0.355(0.723)*	*-0.644(0.52)*	*0.839(0.403)*		*1.002(0.318)*
Illness due to stress						
No	142	36.81(30.36-44.58)	48.12(45.24-50.99)	42.42(39.8-45.04)	140	2.99(2.8-3.19)
Yes	15	31.28(13.24-72.18)	46.62(39.04-54.19)	42.31(35.23-49.38)	15	2.78(2.01-3.56)
t-statistic(p-value)		*0.472(0.638)*	*0.302(0.763)*	*0.025(0.98)*		*0.617(0.538)*
Smoking history						
Non-smoker	89	38.79(30.45-49.36)	47.33(43.73-50.92)	43.55(40.27-46.83)	87	3.01(2.75-3.27)
Ex-smoker	68	33.36(24.73-44.87)	48.86(44.69-53.02)	40.94(37.18-44.7)	68	2.93(2.66-3.21)
t-statistic (p-value)		*0.793(0.429)*	*-0.555(0.58)*	*1.042(0.299)*		*0.405(0.686)*
Alcohol consumption						
<1 unit per week	46	36.04(25.09-51.59)	48.37(42.88-53.86)	40.93(36.28-45.59)	46	3.03(2.7-3.37)
1-21 units/wk	92	31.37(24.46-40.17)	47.35(43.83-50.87)	43.34(40.03-46.64)	92	3.23(2.91-3.54)
>21 units/wk	20	72.0(78.49-106.67)	50.1(43.03-57.17)	41.55(34.89-48.21)	20	3.03(2.21-3.84)
t-statistic (p-value)		***4.244(0.016)****	*0.225(0.799)*	*0.395(0.674)*		*0.35(0.706)*
Underwear						
No boxer shorts	16	31.08(18.33-52.25)	42.06(33.71-50.42)	50.81(41.88-59.75)	16	3.33(2.62-4.04)
Boxer shorts	139	36.18(29.57-44.23)	48.59(45.69-51.48)	41.57(39.01-44.13)	137	2.94(2.74-3.14)
t-statistic (p-value)		*-0.485(0.628)*	*-0.144(0.152)*	***2.265(0.025)****		*1.213(0.227)*
Leisure score index						
<24 points	78	33.49(25.27-44.28)	48.49(44.98-52.01)	42.24(39.06-45.42)	77	3.08(2.82-3.34)
≥24 points	78	40.98(32.17-52.11)	48.01(43.94-52.09)	41.18(38.41-45.95)	77	2.84(2.57-3.12)
t-statistic (p-value)		*-1.085(0.279)*	*0.264(0.859)*	*0.025(0.98)*		*0.435(0.213)*
BMI (kg/m^2)						
<25	74	45.81(36.57-57.33)	49.91(45.77-54.04)	40.67(36.94-44.4)	70	3.12(2.83-3.41)
25-25.99	56	28.25(19.73-40.26)	45.25(40.81-49.69)	45.29(41.32-49.25)	55	2.94(2.62-3.26)
≥30	21	33.96(18.98-60.19)	48.1(40.43-55.76)	41.52(33.99-49.06)	19	2.63(2.18-3.07)
F-statistic		*2.85(0.061)*	*1.16(0.316)*	*1.423(0.244)*		*1.395(0.251)*
Diet						
Meat eater	27	46.8(30.46-71.63)	51.15(43.77-58.53)	40.67(33.9-47.43)	26	2.81(2.26-3.36)
Meat and fish eater	125	34.47(27.85-42.6)	47.79(44.84-50.73)	42.48(39.77-45.2)	124	3.02(2.81-3.23)
t-statistic (p-value)		*1.221(0.224)*	*0.93(0.354)*	*-0.545(0.586)*		*-0.796(0.428)*

Independent t-test and one-way ANOVA F-statistics are shown along with significance (*p*) values. Significant associations are highlighted in bold.*p<0.05, **p<0.01.

Spearman's correlation showed that BMI was negatively correlated with sperm concentration (p=0.035) and total sperm count (p=0.005) (table 4.9). When sperm concentration was restricted to ≥15x10^6/ml, BMI was no longer

correlated with sperm concentration (p=0.604, n=124) and when sperm count was restricted to ≥39x10^6 cells, this was also not significant (p=0.256). %PM and %immotile cells were not correlated with BMI, LSI, alcohol consumption or smoking years when all samples were analysed nor when only samples ≥32% PM or samples <60% immotile were analysed. Seminal volume was not correlated with these lifestyle variables when looking at all data or at samples ≥1.5 ml only.

4.2.1.3.3 Semen parameters and chemical and physical hazards 3 months previous

Sperm concentration was not correlated with any of the chemical or physical hazards when all data points were analysed (table 4.9). However, concentration was significantly lower in men who were exposed to metal dust or fumes (n=24) than men who were not exposed (n=104) when only men with a normal sperm concentration of ≥15x10^6/ml were considered (p=0.018).

Table 4.8 Semen parameters and lifestyle correlations

	Concentration (x10^6/ml)	PM (%)	Immotile (%)	Volume (ml)	Count (x10^6)
BMI					
ρ (p-value)	**-0.171(0.035)***	-0.068(0.406)	0.049(0.548)	-0.136(0.097)	**-0.229(0.005)****
n	**151**	151	151	149	**149**
Alcohol					
ρ (p-value)	**0.164(0.04)***	0.033(0.686)	-0.015(0.853)	-0.006(0.939)	0.136(0.093)
n	**157**	157	157	155	155
LSI					
ρ (p-value)	-0.003(0.974)	0.058(0.469)	-0.062(0.443)	-0.136(0.093)	-0.094(0.246)
n	156	156	156	154	154
Smoking years					
ρ (p-value)	-0.061(0.447)	0.103(0.202)	-0.103(0.202)	-0.037(0.644)	-0.042(0.605)
n	156	156	156	155	155

Spearman's correlation, ρ, and significance (p) values are shown. Significant correlations are shown in bold. *p<0.05, **p<0.01.

%PM sperm, %immotile sperm and seminal volume were not correlated with any of the chemical or physical hazards when all samples were included, nor when samples were restricted to the WHO reference limits (table 2.9).

Table 4.9 Semen parameters and chemical and physical exposures 3 months prior to the study

	n	Concentration	%PM	%immotile	n	Volume
Metal dust or fumes						
No	128	37.65(30.35-46.64)	48.1(45.08-51.11)	42.95(40.2-45.71)	126	3.1(2.86-3.34)
Yes	29	30.94(21.48-44.4)	47.52(41.16-53.87)	37.78(34.33-45.22)	29	3.33(2.71-3.95)
t-statistic (p-value)		*0.802(0.424)*	*0.165(0.869)*	*-0.421(0.674)*		*-0.784(0.434)*
Oils or greases						
No	106	36.36(28.85-45.76)	48.36(45.03-51.7)	41.15(38.18-44.12)	104	3.1(2.83-3.37)
Yes	47	35.95(25.42-40.67)	47.11(42.0-52.21)	45.26(40.52-50.0)	47	3.23(2.78-3.68)
t-statistic (p-value)		*0.054(0.957)*	*0.414(0.68)*	*-1.496(0.137)*		*-0.523(0.602)*
Detergents and soaps						
No	93	35.4(27.5-45.47)	47.61(44.19-51.03)	42.23(39.12-45.35)	91	3.13(2.84-3.42)
Yes	60	38.1(35.83-57.25)	48.28(43.54-43.03)	43.0(38.68-47.32)	60	3.16(2.77-3.55)
t-statistic (p-value)		*-0.372(0.71)*	*-0.236(0.814)*	*-0.293(0.77)*		*-0.128(0.898)*
Glues, adhesives, resins						
No	108	34.86(27.57-44.01)	47.04(43.6-50.48)	42.69(39.54-45.84)	106	3.1(2.82-3.37)
Yes	44	41.13(29.18-57.8)	50.34(45.7-54.98)	41.64(37.5-45.78)	44	3.29(2.86-3.72)
t-statistic (p-value)		*-0.771(0.442)*	*-1.066(0.288)*	*0.373(0.71)*		*-0.742(0.459)*
Paints, varnishes, lacquers						
No	110	36.4(28.79-45.98)	47.89(44.64-51.15)	42.22(39.24-45.2)	108	3.15(2.86-3.43)
Yes	42	35.89(25.69-49.93)	48.5(42.98-54.02)	42.43(37.58-47.28)	42	3.1(2.68-3.52)
t-statistic (p-value)		*0.069(0.945)*	*-0.193(0.847)*	*-0.074(0.941)*		*0.171(0.865)*
Printing inks/dyestuffs						
No	133	35.95(29.35-43.99)	47.69(44.76-50.63)	42.36(39.68-45.03)	131	3.17(2.92-3.43)
Yes	19	38.48(20.42-71.74)	50.63(41.52-59.74)	41.68(33.69-49.68)	19	2.85(2.35-3.36)
t-statistic (p-value)		*-0.231(0.817)*	*-0.689(0.492)*	*0.174(0.862)*		*0.903(0.368)*
Other solvents						
No	113	35.02(27.73-44.13)	47.24(43.94-50.53)	42.59(39.51-45.67)	111	2.99(2.74-3.25)
Yes	38	41.82(30.05-58.02)	51.63(46.83-56.43)	40.37(36.59-44.15)	38	3.58(3.07-4.09)
t-statistic (p-value)		*-0.794(0.428)*	*-1.378(0.17)*	*0.914(0.363)*		***0.577(0.03)****
Heavy vib. machinery						
No	132	36.87(29.95-45.33)	47.24(44.2-50.28)	42.86(40.07-45.66)	130	3.16(2.91-3.41)
Yes	20	32.44(19.07-54.72)	53.5(46.92-60.08)	38.35(33.48-43.22)	20	2.95(2.27-3.63)
t-statistic (p-value)		*0.444(0.657)*	*-1.509(0.133)*	*1.2(0.232)*		*0.605(0.546)*

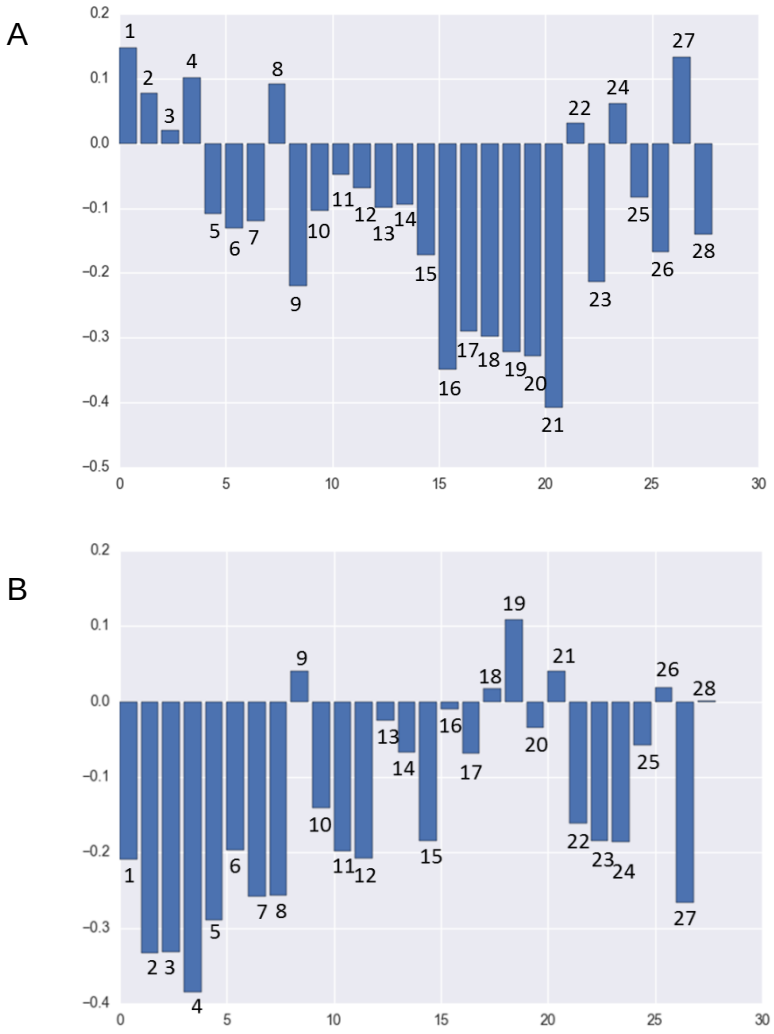
Sig. differences between exposed and non-exposed men were determined using independent t-tests. T-statistics and sig. (*p*) values are shown. Means and 95% CI are shown for each exposure. Sig. results are shown in bold. *$p<0.05$.

4.2.1.3.4 Semen parameters and diet

4.2.1.2.3 Food frequency questionnaire

Twenty-eight food categories were generated from seventy-eight food items on the food frequency questionnaire. Principal components analysis revealed that there were two primary diet types in the population which corresponded to typically “healthy” and “unhealthy” diets. Figure 4.3 illustrates the relative contribution of each food group to both diets with each bar on the graph representing a different group. The contribution (or loading) of each item to the variance is shown by the length of the bars: longer bars contribute a larger amount of variance to the diet. Furthermore, the direction of the bars (positive or negative) indicates food items that are correlating with each other (refer to methods section for further explanation). The healthy diet was typified by consumption of soy, low-fat dairy, all types of vegetables, chickpeas, nuts, cereals, green tea and vitamins while the unhealthy diet was typified by consumption of red, fried and processed meat, fries, butter, high-fat dairy, bread, sugar and confectionary, and caffeinated sugar-sweetened beverages (CSSB). Loading values are shown in table 4.10.

Figure 4.3 Principal components analysis of diet composition



PCA was used to determine the loading of each food group in the "healthy" and "unhealthy" diets, which correspond to the first and second principal components, respectively. Each bar represents a food group or item. The loading of each item on the variance in the data is illustrated by the bar length. Bar direction shows positive or negative correlations in the data. Labelled food groups or items are: chicken/poultry (1), red meat (2), very fried meat (3), processed meat (4), liver (5), fish (6), fried fish (7), fries (8), soy (9), vegetable oil (10), butter (11), high-fat dairy (12), low-fat dairy (13), eggs (14), fruit (15), vegetables (16), cruciferous vegetables (17), green and leafy vegetables (18), chickpeas (19), nuts (20), cereals (21), bread (22), whole grains (23), sugar and confectionary (24), caffeinated non-sugar sweetened beverages (25), green tea (26), caffeinated sugar-sweetened beverages (27), vitamins (28).

Each participant was assigned a "healthy" and an "unhealthy" diet score

which were calculated by multiplying the frequency of consumption of each food group by the item's loading value and taking the sum of these scores. Total variance explained is the amount of variance that this combination of food groups represents in the diet data: the healthy diet represents the largest amount of variance in the data, while the unhealthy diet represents the second largest level of variance.

Table 4.10 Food group loading values for "healthy" and "unhealthy diets

Food group or item	"Healthy"	"Unhealthy"
Red meat	--	0.33
Very fried meat	--	0.33
Processed meat	--	0.38
Fries	--	0.26
Soy	0.22	--
Butter	--	0.20
High fat dairy	--	0.21
Low fat dairy	0.10	--
Vegetables (general)	0.35	--
Cruciferous vegetables	0.29	--
Green and leafy vegetables	0.30	--
Chickpeas	0.21	--
Nuts	0.33	--
Cereals	0.41	--
Bread	--	0.16
Sugar and confectionary	--	0.19
Green tea	0.17	--
Caffeinated sugar-sweetened beverages	--	0.14
Vitamins	0.14	--
Total variance explained	15.57%	12.39%

Food groups not included in the table are chicken, liver, fish, fried fish, vegetable oil, eggs, fruit, whole grains and caffeinated non-sugar-sweetened beverages due to having little effect in both diets (loading <0.1) or having some effect in both diets (loading >0.1). Principal components analysis was used to determine the loading of each food groups on both diets.

The “healthy” and “unhealthy” diets were not correlated with leisure score index, i.e. physical activity levels (p=0.573 and p=0.382, respectively), BMI (p=0.772 and 0.294), or alcohol consumption (p=0.376 and 0.736). Diet scores were categorised into quartiles and semen parameters were analysed for each quartile (table 2.11). Sperm concentration, %PM and semen volume did not show any association with low, low-moderate, moderate-high or high healthy or unhealthy diets. Men in the highest quartile for “unhealthy” diet had significantly lower % immotile sperm (p=0.046) than the men with the lowest quartile. Sperm count was significantly higher in the third quartile of “unhealthy” diet than in the second quartile (p=0.028), however the lowest and higher quartiles were not different from each other. When only samples in the normal range of semen parameters were analysed (table 4.12) there was no significance between “healthy” or “unhealthy” diet scores and semen parameters. There was a gradual increase in sperm concentration, volume and count as men’s “healthy” diet score increased, however all showed a drop in the highest quartile. Conversely, % PM decreased and % immotile sperm increased as men consumed more of a healthy diet, which also reflects the negative association between % immotile sperm and “unhealthy” diet.

Table 4.11 Semen parameters based on "healthy" and "unhealthy" diets

	Healthy					Unhealthy				
	Low (n=39)	Low-Mod (n=38)	Mod-High (n=39)	High (n=38)	F (*p*-value)	Low (n=38)	Low-Mod (n=39)	Mod-High (n=39)	High (n=38)	F (*p*-value)
Sperm concentration ($x10^6$/ml)										
Mean	33.76	38.66	40.35	31.34	0.375	28.96	30.7	46.26	40.03	1.366
95% CI	(21.68-52.28)	(26.17-56.88)	(29.95-54.26)	(20.67-47.26)	(0.771)	(19.3-43.22)	(20.56-45.59)	(31.91-66.89)	(27.74-57.57)	(0.255)
Progressively motile (%)										
Mean	49.79	47.87	46.28	47.74	0.268	44.76	45.31	48.62	53.05	1.894
95% CI	(43.71-55.88)	(42.18-53.56)	(41.26-51.31)	(41.87-53.6)	(0.848)	(38.17-51.36)	(38.9-51.71)	(43.73-53.5)	(49.0-57.1)	(0.133)
Immotile (%)										
Mean	41.28	41.24	43.49	43.5	0.255	46.26	44.0	42.62	36.58	**2.724**
95% CI	(35.68-46.89)	(36.4-46.07)	(38.5-48.48)	(38.29-48.71)	(0.858)	(40.46-52.07)	(38.24-49.76)	(38.06-47.18)	(32.81-40.35)	**(0.046)***
Volume (ml)										
Mean	3.22	3.16	3.46	2.82	1.326	3.08	2.97	3.34	3.29	0.55
95% CI	(2.69-3.75)	(2.82-3.49)	(2.92-4.0)	(2.4-3.24)	(0.268)	(2.69-3.48)	(2.42-3.52)	(2.96-3.72)	(2.74-3.83)	(0.649)
Count ($x10^6$)										
Mean	83.2	98.13	124.43	71.33	1.018	80.83	56.02	142.68	112.71	**3.111**
95% CI	(49.56-139.18)	(60.42-158.96)	(86.9-177.98)	(41.42-122.34)	(0.387)	(52.7-123.68)	(30.24-103.06)	(98.54-206.4)	(73.06-173.58)	**(0.028)***

Means and 95% CIs are shown for semen parameters in each quartile of the "healthy" and "unhealthy" diets. Significant differences between quartiles were calculated using one-way ANOVA; F-statistics and *p*-values are shown for each parameter. Significant correlations are highlighted in bold. *$p<0.05$.

Table 4.12 Effects of diet in men with normal semen parameters

	Healthy	Unhealthy
Sperm concentration (≥15x10^6/ml)	0.824 (0.483)	0.845 (0.472)
Progressively motile (≥32%)	1.448 (0.232)	0.196 (0.899)
Immotile (≥32%)	0.707 (0.549)	0.778 (0.508)
Volume (≥1.5 ml)	1.886 (0.135)	1.261 (0.29)
Count (≥39x10^6)	0.203 (0.894)	0.659 (0.579)

Differences in semen parameters between quartiles of "healthy" and "unhealthy" diets were analysed using one-way ANOVA. F-statistics (*p*-value) are shown.

4.2.1.4 Multivariate analysis of lifestyle and semen parameters

Univariate analysis revealed that sperm concentration was negatively correlated with BMI (*p*=0.035) and positively correlated with alcohol consumption (0.04). Sperm count showed similar but insignificant associations with these variables also. In addition, unhealthy diet score was negatively correlated with % immotile sperm (*p*=0.046) and positively correlated with total sperm count (*p*=0.028). To control for confounding variables that may be influencing these associations, multiple regression analyses were performed.

Due to the evidence of the interactions between diet, BMI, physical activity levels and smoking habits, smoking history (ex-/non-) and LSI were included as control independent variables (IV), alongside ethnicity. Age was not included in the regression analyses as it was positively correlated with BMI (*p*= 0.031) and negatively correlated with having an unhealthy diet (*p*=0.001), and would therefore give rise to multicollinearity in the model, causing redundancy of some variables. In addition, age had not shown any association with semen parameters in the univariate analysis so could be

justifiably excluded. Seminal volume and total sperm count were also controlled for with abstinence time.

The regression models (table 4.13) showed that BMI was significantly negatively correlated with sperm concentration (*p*=0.031) while alcohol consumption was no longer correlated with sperm concentration (*p*=0.166) when controlling for other variables. Overall, the model was not significant due to the lack of predictive power of the other IVs for concentration (*p*=0.058). The association between total sperm count and BMI was less significant in the regression model, decreasing from *p*=0.005 to 0.052, while the effect of alcohol consumption increased in significance slightly from *p*=0.093 to 0.083. However, the overall model was still significant (*p*=0.048). % Immotile sperm was still significantly negatively correlated with having an unhealthy diet in the regression model (*p*=0.018). Ethnicity was an important control variable as it showed significance for both concentration and %immotile sperm.

Table 4.13 Multivariate analysis of the effects of BMI, diet and alcohol on sperm parameters

Dependent variable	Significant IVs in model	Standardised β-coefficient	*p*-value	ANOVA *p*-value
[b]Concentration (x10^6/ml)	BMI	-0.178	**0.031***	0.058
	Ethnicity	0.161	0.054	
[a]Immotile (%)	Unhealthy diet	-0.199	**0.018***	**0.047***
	Ethnicity	-0.161	0.051	
[c]Count (x10^6)	BMI	-0.177	0.052	**0.048***
	Alcohol	0.157	0.083	

A multiple linear regression was run with each semen parameter as the dependent (outcome) variable. Each row corresponds to a different regression model. IVs with sig. <0.1 are shown. Sig. (*p*) values of the overall model are shown in the ANOVA *p*-value column. IVs used in each model were BMI, smoking history, unhealthy diet score, LSI, ethnicity ([a]), plus alcohol consumption ([b]) and abstinence time ([c]).

In a separate multiple regression, the effect of solvents on semen volume, which was originally found to have a positive correlation (*p*=0.03) was

controlled for abstinence time. The regression removed the significance of solvents on volume (p=0.097).

To summarise, the multivariate analyses showed that higher BMI was associated with lower sperm concentration and sperm count, while those who consumed high amounts of alcohol had higher sperm counts. Finally, men with an unhealthy diet had lower levels of immotile sperm.

4.2.2 Semen parameters and ART outcomes

4.2.2.1 Semen parameters in IVF and ICSI patients

The effect of different semen parameters on the outcome of IVF and ICSI treatment was investigated. During ART mature and motile sperm are

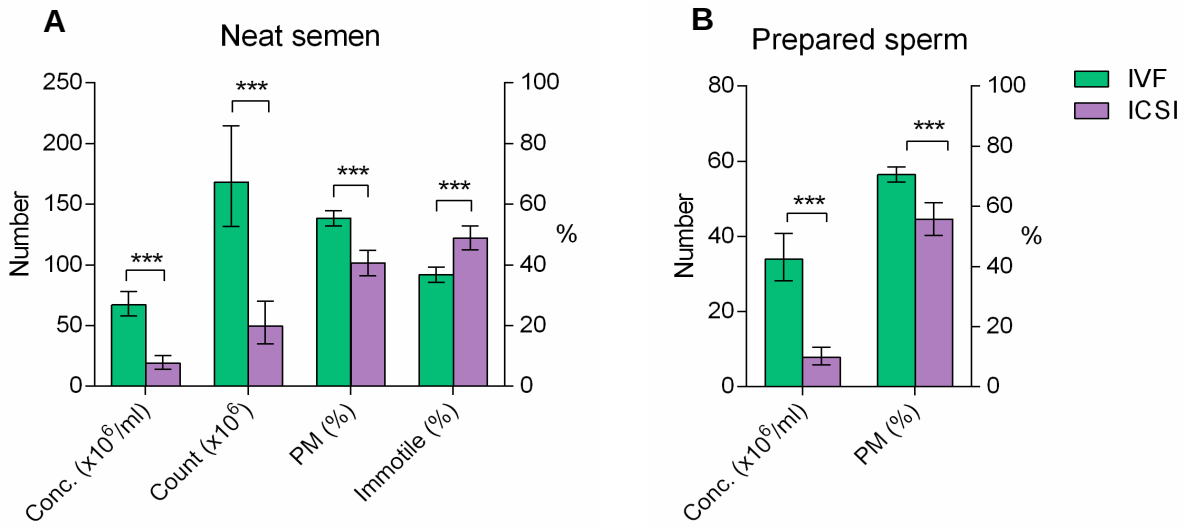


Figure 4.4 Sperm parameters in IVF and ICSI patients

Sperm concentration, count and % PM were higher while % immotile cells were lower in neat semen in IVF patients (A). Following preparation, sperm concentration and % PM were still higher in IVF than in ICSI patients (B). Error bars represent upper and lower 95% CIs. Significant results of independent t-tests are shown. ***p<0.001.

separated from the seminal plasma and washed in media prior to fertilisation; this fraction is known as prepared sperm. The standard of neat semen parameters before preparation may convey the likelihood of ART outcomes as it reflects the quality of the sample as a whole. Following preparation, although the better quality sperm have been isolated, sperm quality may still reflect ART outcomes.

In this study, 50.9% of patients underwent IVF treatment while 48.4% underwent ICSI. Neat semen parameters were all higher in the IVF than the ICSI group. Sperm concentration, % PM cells and total sperm count were significantly higher and %immotile cells significantly lower in the IVF group (fig. 4.4 A). This is reflected in the fact that there were a higher percentage of infertile men in the ICSI group (fig 4.5 B) (76.62%). Following preparation of these neat samples, sperm concentration and % PM remained significantly higher in the IVF than the ICSI group (fig. 4.4 B). Centrifugation of sperm cells results in hyperactivation, which manifests in an increase in forward progressive motility; this can be seen as the increase in %PM from neat to prepared sperm (fig. 4.4 A and B). In both neat semen and in prepared sperm, concentration was highly positively correlated with %PM ($p<0.001$). Due to the stark differences in semen quality in IVF and ICSI patients, ART outcomes for these groups were analysed separately.

4.2.2.2 ART outcomes and demographics for IVF and ICSI patients

Overall ART outcomes in the IVF and ICSI groups were compared along with primary factors that can affect ART outcomes including the egg stimulation protocol (L D21 or antagonist), duration of stimulation (days) and the day of transfer of embryos into the female (fig. 4.5 A). Fertilisation and cleavage rates were not significantly different between the IVF and ICSI groups. Of those patients who had successful fertilisation and cleavage, and following transfer of the embryos, pregnancy rates were slightly higher in the ICSI group (50%) compared to the IVF group (42.9%) however this was not statistically significant (p=0.401).

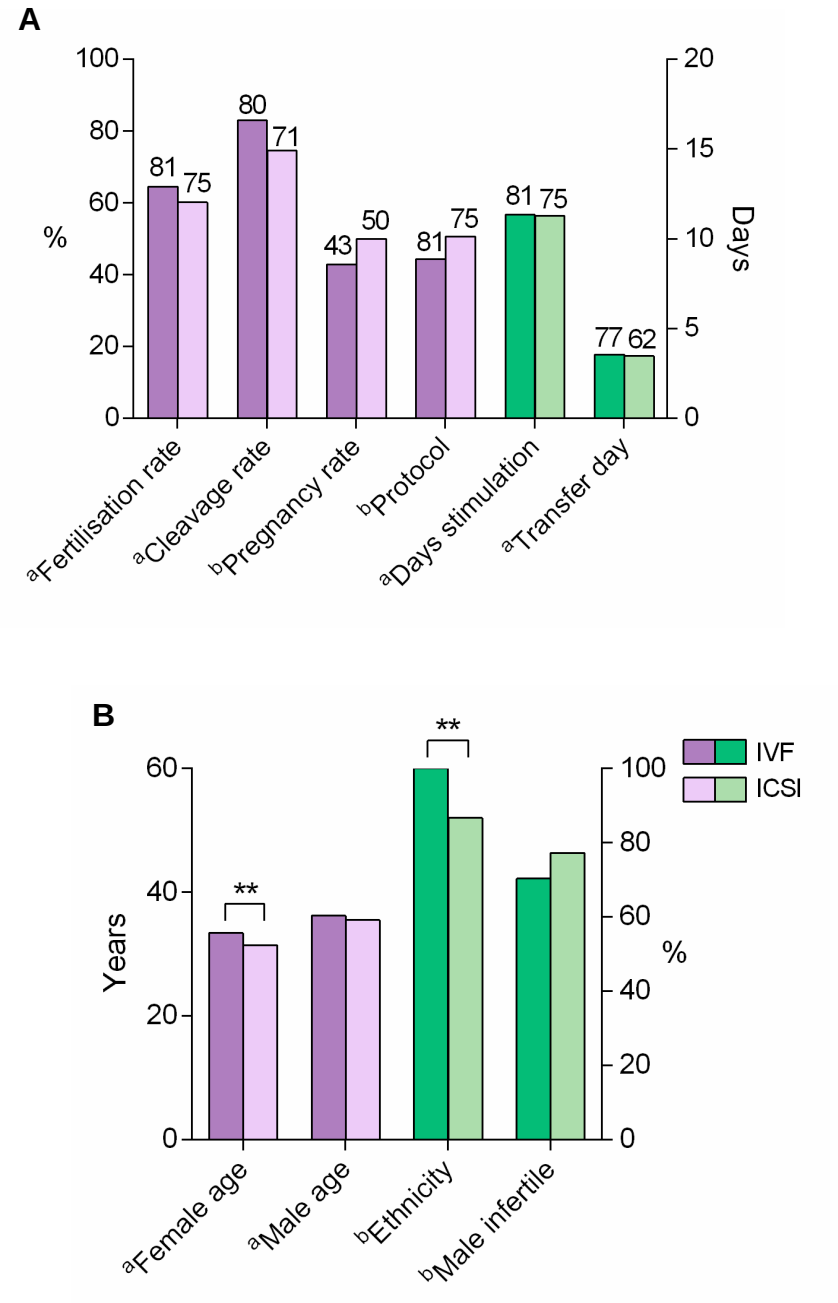


Figure 4.5 Outcomes and demographics of IVF and ICSI treatment

Figure A shows means for fertilisation rates, cleavage rates, days of stimulation and transfer day, while showing the %successful pregnancies and %patients who underwent the L D21 protocol as a pose to the antagonist protocol. Figure B shows means for female and male age, ethnicity as %White British men and also %infertile men. Significant differences between IVF and ICSI groups were calculated using the Mann-Whitney U test for continuous variables ([a]) and χ^2 for categorical variables ([b]). Total numbers of patients are shown for each bar (A). Total number of IVF and ICSI patients was 81 and 75, respectfully (B). Purple and green bars correspond to scales on left and right y-axes respectively. ***p*<0.01.

Female age was significantly higher in the IVF group than the ICSI group (p=0.005) while male age was not different between the groups (fig. 4.5 B). All men in the IVF group were White British, while in the ICSI group 12.99% were of another ethnic background. There was no statistically significant difference in the number of infertile men between the two groups.

4.2.2.3 Univariate analysis of sperm parameters with IVF and ICSI outcomes

Associations between sperm quality and ART outcomes in IVF and ICSI patients were investigated using Spearman's correlation and the Mann-Whitney U test (table 4.14). Sperm concentraiton, %PM and total sperm count were positively correlated with fertilisation rate (p<0.05). Concomitantly, % immotile cells were negatively correlated with fertilisation rate (p<0.05). As there was multi-collinearity between the semen parameters (p<0.001), it was necessary to try to identify the main impacting factor. Total

Table 4.14 Correlations between semen parameters, fertilisation, cleavage and pregnancy rates in IVF and ICSI patients

	IVF (p-values)			ICSI (p-values)		
	Fertilisation rate[a] (%) (n=81)	Cleavage rate[a] (%) (n=80)	Pregnancy rate[b] (%) (n=77)	Fertilisation rate[a] (%) (n=75)	Cleavage rate[a] (%) (n=71)	Pregnancy rate[b] (%) (n=62)
Concentration (x10^6/ml)						
Neat	0.352	0.419	0.247	**0.021***	0.988	0.933
Prepared	0.233	0.779	0.703	0.075	0.72	0.746
Progressive motility (%)						
Neat	0.92	**0.049***	0.849	**0.031***	0.356	0.335
Prepared	0.845	0.662	0.829	0.632	0.815	0.234
Immotile (%)						
Neat	0.55	0.101	0.853	**(-)0.047***	0.222	0.149
Count (x10^6)						
Neat	0.139	0.479	0.294	**0.026***	0.876	0.622

Associations between semen parameters and ART outcomes were analysed using Spearman's correlation ([a]) for continuous variables and Mann-Whitney U test for categorical variables ([b]). Significant results are shown in bold. All semen parameters were correlated with fertilisation rates in ICSI samples. *p<0.05.

sperm count is a function of sperm concentration and is also dependent on seminal volume which is highly variable, so this can be excluded. % PM is considered to be a more informative marker of semen quality than % immotile cells, as % immotile sperm is a direct opposite measurement to % total motility. Therefore, sperm concentration and sperm % PM in neat semen are associated with fertilisation rates and were considered for multivariate analysis.

4.2.2.4 Multivariate analysis of sperm parameters with IVF and ICSI outcomes

As sperm concentration and % PM in neat semen was positively correlated with fertilisation rates in ICSI patients, the next step was to control for other factors that could influence the ART outcomes. Therefore, a multiple linear regression analysis was carried out to control for protocol type, days of stimulation, female and male age, attempt number and ethnicity.

Table 4.15 Multiple regression analysis of sperm parameters and fertilisation rates in ICSI patients

Model	Parameter	Unstandardised B-coefficient	Standardised β-coefficient	*p*-value	ANOVA *p*-value
1	Concentration (x10^6/ml)	11.382	0.227	0.051	0.131
2	% PM	0.339	0.239	**0.042***	0.116

Regression analysis was performed for concentration and % PM on fertilisation rates in two separate models while controlling for female age, ethnicity, attempt, protocol and days of stimulation. Unstandardised B and standardised β values are shown. *p*-values indicate the significance of each independent variable on fertilisation rates while controlling for all other variables. Standardised β values represent the number of SD changes expected in the outcome variable for 1 SD change in the independent variable, while all other factors are held constant. Unstandardised B values are the coefficients in their original units. *$p<0.05$.

The multivariate analysis showed that in ICSI patients sperm concentration was borderline significantly associated ($p=0.051$) and % PM was significantly

associated (*p*=0.042) with fertilisation rates (table 4.15). The two parameters were run in separate regression models as they were highly correlated with each other, and this can lead to redundancy in the results. The strength of a variable's effect can be interpreted from the standardised β-values: both variables had the highest β-coefficients in the models, with ethnicity also having some influence (β=-0.223 and -0.235 for model 1 and 2, respectively). Male age was initially included in the regression, however it had an extremely low β-value and was highly correlated with female age; this justified its removal. Female age did not show a significant effect in model 1 (*p*=0.119) or 2 (*p*=0.171). None of the other variables were significant or borderline significant.

Multiple regression analysis was also performed to confirm the association of % PM with cleavage rates in IVF patients. Table 4.16 shows that the significance of % PM, whilst controlling for all other factors, actually increased from 0.049 to 0.023. No other independent variables showed a significant effect on cleavage rates. Ethnicity was not included in the analysis as all IVF participants were White British. Standardised β-coefficients showed that % PM has the greatest weighting, while attempt and protocol type also had strong negative effects on cleavage rate. The overall significance of the model was 0.09.

Table 4.16 Multiple regression analysis of % progressive motility in neat semen and cleavage rates in IVF patients.

	Unstandardised B-coefficient	Standardised β-coefficient	*p*-value	ANOVA *p*-value
PM (%)	0.556	0.258	**0.023***	
Female age (years)	0.582	0.097	0.387	
Attempt	-9.02	-0.134	0.235	0.09
Protocol (L D21/antagonist)	-7.285	-0.146	0.188	
Stimulation (days)	0.564	0.045	0.685	

Unstandardised B and standardised β values are shown. *p*-values indicate the significance of each independent variable on fertilisation rates while controlling for all other variables. Progressive motility (%) was positively correlated with fertilisation rates. *$p<0.05$.

To summarise the effects of semen parameters on ART outcomes, sperm concentration and % PM were positively correlated with fertilisation rates in ICSI patients, however these were not significant. Furthermore, % PM was highly correlated with cleavage rates in IVF patients. Prepared sperm parameters were not associated with ART outcomes in ICSI or IVF patients.

4.2.3 Lifestyle and ART outcomes

4.2.3.1 Univariate analysis of lifestyle factors and ART outcomes

To investigate the effects of lifestyle on ART outcomes, univariate analyses were firstly carried out between lifestyle factors and fertilisation, cleavage and pregnancy rates. One-way ANOVA and Tukey post-hoc tests were used to identify differences between categorical variables (table 4.17). Univariate analysis revealed that having an unhealthy diet was significantly negatively

Table 4.17 Univariate analysis of lifestyle factors and ART outcomes in IVF and ICSI patients

IVF		ICSI	
Fertilisation rate			
BMI (n=77)		BMI (n=70)	
≤23.1	61.03(49.97-72.08)	≤23.1	65.44(51.14-79.74)
23.11-24.65	72.06(62.46-81.66)*	23.11-24.65	74.47(62.28-86.66)*
24.7-26.47	68.09(58.84-77.35)	24.7-26.47	59.76(50.18-69.34)
>26.48	52.93(39.67-66.19)*	>26.48	51.77(39.9-63.64)*
p-value	0.065	*p*-value	0.057
Cleavage rate			
Units alcohol/wk (n=77)		Unhealthy diet (n=68)	
0	73.26(58.75-87.76)*	Low	91.19(82.04-100.34)*
0.1-7.8	88.87(79.43-98.3)	Low-mod	72.04(57.23-86.86)
7.81-15.6	77.87(65.94-89.81)	Mod-high	49.02(20.6-77.43)*
≥15.61	92.06(84.14-99.98)*	High	73.3(54.6-92.0)
p-value	0.061	*p*-value	0.008**

Continuous lifestyle variables were categorised into quartiles and analysed by one-way ANOVA for fertilisation and cleavage rates. IVF and ICSI patients were analysed separately. Significance values below 0.1 are reported. Tukey post-hoc tests revealed which categories were different, shown as flagged (*). ** $p<0.01$.

correlated with cleavage rates in ICSI patients (p=0.008). Variables which showed p-values of <0.1 for association with fertilisation, cleavage and pregnancy rates were BMI, alcohol consumption and physical activity levels (LSI) (table 4.18). Results which showed a significance of at least 1.0 were included in a multivariate analysis. Other lifestyle factors were not associated with ART outcomes.

4.2.3.2 Multivariate analysis of lifestyle and ART outcomes

When controlling for female age, duration of stimulation, protocol and attempt, BMI and alcohol consumption were no longer correlated with fertilisation and cleavage rates in IVF patients, respectively (p=0.365, p=0.105). The significant univariate association between unhealthy diets and lower cleavage rates in ICSI patients was also no longer significant when controlling for other factors (p=0.109). However, the main finding was that BMI was significantly negatively correlated with fertilisation rates in ICSI patients (p=0.044) (table 4.19). In this regression analysis, % PM was also added to the control variables as previous analyses showed that % PM was slightly positively correlated with fertilisation rates (section 4.2.4). Sperm concentration was not included in the regression as multicollinearity between these variables (p=0.06) can cause redundancy. % PM and BMI were not correlated (p=0.943). Overall, increased BMI was linked to lower fertilisation rates, when controlling for other factors; ethnicity was also negatively correlated and % PM was positively correlated with fertilisation rates. Logistical regression was performed to investigate the likelihood of pregnancy outcome with an increase or decrease in physical activity (LSI). These factors were not significantly associated when controlling for female age, stimulation, protocol and attempt.

Table 4.18 Univariate analysis of LSI with pregnancy rate in IVF patients

LSI (n)	Pregnancy rate	*p*-value
Low (20)	65%	0.064
Low-mod (13)	38.4%	
Mod-high (18)	44.4%	
High (24)	25%	

χ^2 analysis for categorical variables was carried out to identify differences in pregnancy rates between LSI quartiles.

Table 4.19 Multiple regression analysis of BMI and fertilisation rates in ICSI patients

	Unstandardised B-coefficient	Standardised β-coefficient	*p*-value	ANOVA *p*-value
BMI (kg/m^2)	-4.954	-0.228	**0.044***	
Female age (years)	-1.003	-0.171	0.145	
Stimulation (days)	1.224	0.098	0.403	
Protocol (L D21/antagonist)	0.837	0.017	0.885	**0.014***
Attempt	0.971	0.015	0.901	
Ethnicity	-18.573	-0.246	**0.039***	
%PM	0.329	0.241	**0.035***	

Unstandardised B and standardised β values are shown. *p*-values indicate the significance of each independent variable on fertilisation rates while controlling for all other variables. BMI, ethnicity and progressive motility (%) were correlated with fertilisation rates. *$p<0.05$.

4.3 Discussion

This study investigated the associations between lifestyle factors and semen parameters in male partners of couples presenting to a fertility clinic. The study also looked at IVF and ICSI outcomes in the study population to determine whether semen parameters were an important factor in successful fertilisation, cleavage and pregnancy rates, and whether male lifestyle influenced this. After controlling for confounding variables, the results showed first and foremost that BMI was strongly negatively associated with sperm concentration and sperm count. Furthermore, sperm concentration was slightly positively correlated with alcohol consumption, while having an unhealthy diet was negatively correlated with the percentage of immotile sperm. % progressive motility of sperm from neat semen was associated with higher cleavage rates in IVF patients, while neat semen concentration and progressive motility were associated (insignificantly) with higher fertilisation rates in IVF patients. Finally, BMI was also associated with lower fertilisation rates in IVF patients.

BMI was the only lifestyle variable found to be consistently negatively associated with sperm concentration throughout the various statistical analyses, which corroborates other findings (Sermondade *et al.,* 2013; Chavarro *et al.,* 2010; Luque *et al.,* 2015). However, due to the number of studies that have found no association between BMI and sperm quality, the causal link remains inconclusive (MacDonald *et al.,* 2013). Additionally, BMI was not associated with sperm concentration above $15x10^6$/ml suggesting that this may primarily affect men with substandard sperm quality. Inclusion of other estimates of body adiposity, such as waist circumference, have found an association with lower sperm volume and sperm count and BMI (Eisenberg *et al.,* 2014), and there is evidence that weight loss intervention can improve sperm DNA integrity (Faure *et al.,* 2014). Notably, this latter weight loss study found changes in abdominal fat without changes in BMI, which again highlights the inadequacy of BMI as a marker of health. Male obesity can lead to a lower likelihood of achieving successful pregnancy within 12 months of attempting (Ramlau-Hansen *et al*., 2007), however the

consequences for ART outcomes are inconclusive. Two meta-analyses published in 2015 found conflicting results: Le *et al.* (10 studies) found no association between BMI and pregnancy rates while Campbell *et al.* (30 studies) found that higher BMI was linked to lower live birth rate; importantly, both studies found no differences in semen parameters. Anifandis *et al.* (2013) found a reduction in embryo quality with male BMI which was also independent of semen parameters. These studies collectively suggest that the sperm quality and ART outcomes may be independently affected by BMI. The result presented here found that higher male BMI was associated with lower fertilisation rates in ICSI patients. This cannot be attributed to poor standard semen parameters as sperm are injected into the oocyte for ICSI. As sperm concentration was lower in men with higher BMI this may indicate some error in spermatogenesis. BMI can affect hormonal regulation without affecting standard sperm parameters (Al-Ali *et al.,* 2014), suggesting that sperm may be affected at a molecular or genetic level instead which is preventing successful fertilisation. The major drawback of our finding is that female BMI was not available and therefore could not be controlled for, which is known to affect egg quality and fertilisation rates (Shah *et al.,* 2011). Further investigations controlling for these factors are necessary to fully conclude that male BMI is indeed associated with reduced fertilisation rates in IVF.

How sperm quality changes with advancing age has been a topic of ongoing investigation. Research has shown that advancing male age is associated with compromised genomic integrity (review in Sharma *et al.,* 2015), yet this is not always accompanied by changes to semen parameters (Nijs *et al.,* 2011). In this study, male age was not correlated with any of the semen parameters analysed. Although this corroborates some studies, a recent thorough meta-analysis of 90 studies concluded that male age was associated with decreases in semen volume, total sperm count, % total motility, % progressive motility, % morphologically normal sperm and also with an increase in DNA fragmentation (Johnson *et al.,* 2015). The lack of association found in this study may be due to the relatively small range of ages (23-57 years) under investigation (mean=36 years) which may not

reveal the true extent of the trends. With regard to IVF and ICSI outcomes in this study, male age had no effect on fertilisation, cleavage or pregnancy rates, which supports the findings of existing studies (Nijs *et al.,* 2011; Begueria *et al.,* 2014; Wu *et al.,* 2015), although some evidence remains that there is a paternal age effect, particularly over 40 years (Kidd *et al.,* 2001; de La Rochebrochard *et al.,* 2006). Maternal age remains the most important prognostic factor for ART outcomes (ESHRE Capri Workship, 2005; Hourvitz *et al.,* 2009).

The effect of modifiable lifestyle factors on sperm quality and ART is important to elucidate as simple lifestyle changes could improve reproductive outcomes. This study investigated unhealthy and healthy diets in relation to semen parameters and IVF and ICSI outcomes. The two diets were similar to those identified elsewhere of men in the United Kingdom which verifies the PCA analysis (Slimani *et al.,* 2002). Men who had a more unhealthy diet consisting of red, fried and processed meat, fries, butter, high-fat dairy, bread, sugar and confectionary, and CSSBs, had lower levels of % immotile sperm. This is the opposite trend to what would generally be expected from an unhealthy lifestyle and contrasts directly to the results of one study in which a "prudent" diet typified by consumption of fish, chicken, fruit vegetables and whole grains was linked to higher levels of % progressive motility (Gaskins *et al.,* 2012). In this study, Gaskins *et al.* recruited participants from a young healthy men's study, rather than from an infertility clinic, suggesting that diet types may show different trends in the general population. This issue was confronted by analysing diet trends in men with at least one normal semen parameter, in which case having an unhealthy diet was no longer linked to lower % immotile sperm. The healthy diet was associated with increases in sperm concentration, volume and count up to the moderate-high group, but then dropped in the highest group. One explanation for this could be that some products associated with the healthy diet such as soy, can be damaging to sperm due to the phyto-estrogens present. Phyto-estrogens are plant-derived endocrine disruptors which mimic oestrogen hormone activity by binding to the oestrogen receptor and have been linked to lower sperm concentration, but not motility, in a study also

looking at men attending a fertility clinic (Chavarro *et al.,* 2008). However, elsewhere phyto-oestrogens have been shown to have no effect on sperm parameters or hormone levels (Mitchell *et al.,* 2001).

It is difficult to firmly conclude the causal effects of specific food items on sperm parameters as individual diets vary so widely, which is why this study focussed on general diet trends. However some studies have attributed specific foods to changes in semen parameters, including sugar sweetened beverages, trans-fatty acids, fish and the breadth of research on anti-oxidant containing foods (Chiu *et al.,* 2014; Chavvaro *et al.,* 2014; Afeiche *et al.,* 2014; Zareba *et al.,* 2013). There are few studies investigating the effects of male diet on ART outcomes but Xia *et al.* (2015) found that increased consumption of processed meat is associated with lower fertilisation rates. Although processed meat was one of the foods with the largest loading in the unhealthy diet, this study did not find an association between unhealthy lifestyles and IVF or ICSI outcomes.

A point of consideration with regard to grouping the diets as “healthy” and “unhealthy” is that the other lifestyle factors in the analysis did not fit with typical healthy or unhealthy lifestyles, i.e. there was no association between healthy and unhealthy diets and BMI, physical activity or alcohol consumption. Therefore those individuals with unhealthier diets were not sharing the traditional attributes of being unhealthy, i.e. being overweight or obese, having low activity levels and drinking more alcohol. BMI itself was also not related to physical activity levels, calling into question the reliability of the self-reported data in the questionnaire.

The benefits of being physically active for human health are extensive. LSI was calculated from the number of times per week participants carried out moderate and strenuous exercise for more than 15 minutes, which was designed and is still commonly used as a method for estimating physical activity levels (Godin and Shephard, 1985). LSI scores were analysed in terms of being active or insufficiently active, as well as on a continuous scale, and no correlation was identified between physical activity levels and semen parameters. This result is not surprising as in the current literature,

like BMI, there is a disparity as to whether and to what extent physical activity affects semen parameters. Two similar studies on young healthy men found contrasting results, with one reporting no effect and another reporting a significant improvement in sperm quality with physical activity on a continuous scale (Gaskins *et al.,* 2014; Minguez-Alarcon, *et al.,* 2014). In comparison, men who participated in a large amount of sedentary behaviour (measured as TV watching) had lower sperm concentration (Gaskin *et al.,* 2015). There is evidence that the type of physical activity men engage in can have different bearings on sperm quality, which was not accounted for in this study, and may be one of the reasons for no differences being detected (Gaskin *et al.,* 2014). A more likely reason for no association being found in this study may be due to the subfertility of the study population, in which case any small changes in sperm quality due to physical activity may be negligible. However, when LSI was analysed only in men with semen parameters above the WHO recommendations for a normal semen sample, there was still no association. Gaskins *et al.* (2014) also found that paternal physical activity was not associated with clinical outcomes of ART, which is also the case in this study.

Curiously, alcohol consumption was associated with higher sperm concentration and sperm count in the univariate analyses, with the greatest increase in sperm concentration being in men who consumed more than the recommended guidelines of 21 units per week. When controlling for confounding variables, these were no longer significant. Despite alcohol being linked to low semen quality in healthy men (Jensen *et al.,* 2014), Povey *et al.* (2012) also found that men who consumed alcohol in the 3 months prior to their study had lower likelihood of low-motile sperm concentration. This was not significant following multivariate analysis, however when considered in conjunction with the results from this study, which provided more detail in terms of the quantity of alcoholic units consumed per week, it suggests that there may be more to the thus far superficial information on alcohol and semen parameters. It is possible that certain personality traits that define a man's drinking habits may also influence other lifestyle choices, through which moderate alcohol

consumption could lead to improved semen parameters. Paternal alcohol consumption was associated with decreased live birth rates and increased miscarriage rates in couples undergoing IVF treatment (Klonoff-Cohen *et al.,* 2003), yet no trend was seen between alcohol consumption and IVF or ART outcomes in this study.

The effects of current smoking could not be investigated on this cohort of men as smoking is prohibited during ART treatment. Therefore, men reported to be either ex-smokers or non-smokers and no difference was found in any of the semen parameters between the non- and ex-smoking groups. This is in accordance with other findings related to men attending fertility clinics (Pacey *et al.,* 2014; Povey *et al.,*2012). The duration of smoking (years) was also investigated to determine if long-term smoking could have an effect on fertility, however this also did not show any association with semen parameters. If men had stopped smoking more than 3 months before giving their sperm sample, any smoking-induced damage may not have manifest in the present sperm, as a full round of spermatogenesis would have completed in that time. A more insightful variable to consider would be whether men had been exposed to maternal smoking *in utero* as this is known to drastically affect sperm and fertility of male offspring (Axelsson *et al.,* 2013) and the extent of this damage has been shown in controlled animal studies (Sobinoff *et al.,* 2014). Correct germ cell development is crucial for a man's adult fertility as spermatogonia are the source of mature sperm post-puberty. At this vulnerable stage sperm would therefore be affected by exposure to toxins such as tobacco smoke. Thus, it would be insightful to ask men about their mother's smoking habits while pregnant as this could describe a large proportion of the variation in semen quality seen here. Smoking was also not associated with IVF or ICSI outcomes in this study.

Men attending the fertility clinic were advised to wear loose fitting underwear and were asked in the questionnaire whether they wore boxer shorts. The results showed that wearing boxer shorts was associated with a lower number of immotile sperm, which supports a previous study which found that wearing boxer shorts was associated with better low-motile sperm

concentration (Povey *et al.,* 2012). This supports evidence that suggests that tight underwear can impair testicular function due to elevated scrotal temperatures, however the imbalance of numbers of men reporting to wear boxer shorts (n=139) versus not wearing (n=16) could suggest a false positive result here. Furthermore, some participants in the “no boxer shorts” group reported to wear no underwear at all which would not present the same risk as tight boxers. These results should therefore be interpreted with caution.

Chemical and physical exposures were not found to be associated with semen parameters in this study, which is in accordance with Jurewicz *et al.* (2014) who found that only exposure to high levels of noise was linked to poorer semen parameters, while sitting, exposure to PVC and high temperatures were not significantly associated. Notably, exposures which did not result in a change in semen parameters did show an increase in DNA fragmentation, indicating that many of these modifiable lifestyle factors are having damaging effects at the DNA rather than the morphological level. Other studies have shown significant effects of chemical and physical exposures, including lower sperm quality with heavy metals and solvents exposure, and specifically lower sperm motility associated with vibrating machinery and hot environments (De Fleurian *et al.,* 2009).

Exposure to pesticides, herbicides, fertilisers, dry cleaning fluids, PVC or plasticisers, extremely hot environments, non-ionizing radiation, X-rays or driving for long periods were not analysed due to low participant numbers. However, some of these have previously been shown to affect semen parameters: De Fleurian *et al.* found that hot environments were linked to lower sperm motility while Chiu *et al.* (2015) found an association between poor sperm morphology and pesticide-contaminated foods and Kumar *et al.* (2013) found poor sperm morphology in men exposed to ionisation radiation, i.e. X-rays. Sperm morphology was not analysed in this study, but seems to be an important parameter to measure for sperm quality.

In this study, IVF and ICSI patients both represented roughly half of the study population. Semen parameters were all significantly poorer in the ICSI group

as expected; ICSI treatment involves the selection of a single sperm for injection into the oocyte usually due to very poor semen parameters that prevent fertilisation in conventional IVF. There was no difference in fertilisation, cleavage or pregnancy rates between IVF and ICSI groups, which is agreeable with existing research that shows that once embryos have reached the transfer stage, there is no difference in pregnancy outcome based on treatment type although embryo quality can differ (van der Westerlaken *et al.,* 2006). Sperm quality was associated with some ART outcome measures. Increased sperm concentration and % PM in neat semen were both associated with higher fertilisation rates in ICSI patients which has been shown elsewhere (Lu *et al.,* 2012; Loutradi *et al.,* 2006). These two variables are also highly correlated with each other so it is difficult to distinguish which is the dominant factor. As ICSI involves sperm injection, the low fertilisation rates cannot be due to failure of sperm to successfully travel to or penetrate the egg plasma membrane. It is therefore probable that other mechanisms, possibly molecular or genetic, can interrupt fertilisation, such as improper expression or activity of phospholipase C zeta (PLCζ). PLCζ is a sperm factor released into the egg upon fertilisation which activates the egg by initiating intracellular calcium oscillations (Kashir *et al.,* 2010). Increased BMI was also found to be associated with lower fertilisation rates in these ICSI patients. As BMI and sperm concentration are correlated with each other, it is difficult to know which is causing the change in fertilisation rates. Again, as female BMI or other female lifestyle factors were not controlled for, these findings require further validation. Finally, in IVF patients, higher % PM in neat semen was associated with higher cleavage rates.

4.3.2 Conclusion

In conclusion, some modifiable environmental and lifestyle factors may influence sperm parameters, which could have consequences for IVF and ICSI, but male lifestyle alone has little bearing on ART outcomes. As others have suggested (Povey *et al.* 2012), although some lifestyle changes may

lead to small improvements in semen quality, it is unlikely that this will occur to an extent that the chances of successful ART are increased.

Chapter 5

An *in vitro* model to investigate the effects of glycation on human sperm

5.1 Introduction

Lifestyle habits known to be detrimental to health include poor diet, obesity, sedentary behaviour and smoking. The non-enzymatic glycation of proteins, lipids and nucleic acids by reducing sugars to form stable covalent advanced glycation end products (AGEs) is thought to be one mechanism behind the progression of disease states as a result of these damaging lifestyle factors. AGE formation on biomolecules occurs as a result of the reaction of the carbonyl (C=O) group of reducing sugars with the amine (NH_2) group of proteins, lipids or nucleic acids through the Maillard reaction. Glycation is accelerated in hyperglycaemic conditions and AGEs accumulate in the body over time, implicating AGEs in many diabetes- and age-related diseases. AGEs are also formed exogenously: cigarette smoking is a source of toxic AGEs that leads to an accumulation of AGEs in the tissues of smokers (Cerami *et al.,* 1997; Nicholl *et al.,* 1998); while high-temperature cooking of foods, particularly meats, is a dietary source of AGEs (Goldberg *et al.,* 2004). Several lifestyle traits and environmental exposures can therefore contribute toward increasing levels of AGEs in the body.

A decline in male fertility over recent decades has been reported in several studies looking at changes in semen parameters (Auger *et al.,* 1995; Carlsen *et al.,* 1992; Rolland *et al.,* 2013; Romero-Otero *et al.,* 2015). AGEs have been investigated as a potential contributor to this decline in sperm function

as one of the major AGEs, carboxymethyl-lysine (CML) has been found elevated on the sperm of diabetic men (Karimi *et al.,* 2011; Mallidis *et al.,* 2009). CML is a glycoxidation product: it is formed on proteins by a combination of glycation and oxidation reactions (Fu *et al.,* 1996). The major mechanisms of damage by AGEs are in altering protein structure and function, crosslinking extracellular matrix (ECM) proteins, and activating the inflammatory response in cells on interaction with the receptor for AGE, RAGE (Goldin *et al.,* 2006; Ahmed, 2005; Zhang *et al.,* 2006; Guimaraes *et al.,* 2010). AGEs are also a potent source of reactive oxygen species (ROS), and as sperm are particularly susceptible to oxidative damage, elevated ROS levels can contribute to poor sperm function (Sharma *et al.,* 1999; Aziz *et al.,* 2004; Agarwal *et al.,* 2014b).

Research suggests that AGEs could be a source of damage to sperm. Firstly, higher levels of seminal plasma AGEs correlate with sperm lipid peroxidation levels, indicating a direct role for the action of AGE-generated ROS (Karimi *et al.,* 2011). Secondly, RAGE is located on sperm cells as well as throughout the testis and epididymis, suggesting that the inflammatory response pathway could be a mechanism for ROS generation in sperm and in surrounding cells. Interestingly, higher levels of RAGE are found in diabetics than non-diabetics (Mallidis *et al.,* 2007) and this directly correlates with nuclear DNA fragmentation though it does not reflect changes in standard semen parameters (Karimi *et al.,* 2012). It seems that AGE-mediated damage could occur as far as the DNA level without showing differences in sperm concentration, motility or morphology. This is important to consider as infertility is defined clinically by these features only.

The occurrence of AGE and RAGE in healthy males shows that these compounds have some baseline effect on sperm function, and may cause oxidative stress, lipid peroxidation and sperm DNA damage. It is important to illustrate the mechanistic action of AGEs on these markers of sperm damage, and this can be achieved through the use of *in vitro* cell assays. Many *in vitro* glycation assays have been carried out on different cell types as well as on purified proteins to elucidate causes and consequences AGE-

mediated damage (Portero-Otin *et al.,* 2002; Nass *et al.,* 2010; Ahmed *et al.,* 2005b). An *in vitro* assay for human sperm will provide some evidence for whether these compounds are an important factor to consider in regards to infertility, as has been achieved with oocytes (Liu *et al.,* 2013). Importantly, as AGE levels are related to lifestyle and environment, AGE-mediated sperm damage could be reduced through lifestyle interventions.

5.1.1 Aims

1. To develop an *in vitro* cell-based assay to investigate the effects of glycation on sperm function.
2. To measure CML formation in glycated sperm and determine the impacts on ROS generation, oxidative DNA damage and HA binding capacity.
3. To investigate differences in CML levels between obese men compared with normal weight men.
4. To determine the effects of *in vitro* glycation of the hyaluronidase protein.

5.2 Results

5.2.1 Formation of AGEs on sperm glycated *in vitro* over 6 days

5.2.1.1 Glucose does not glycate sperm *in vitro*

Glucose sugar was first utilised as the glycating agent for *in vitro* incubation with prepared sperm for 6 days at 37°C. The formation of the prominent AGE, carboxymethyllysine (CML), was measured in sperm glycated in the presence of SPM and glucose at 30 and 50 mM (fig. 5.1). As sperm are non-dividing cells, they can be maintained in culture for a limited period of time. The number of days that sperm could be kept in culture in the presence of various compounds was determined based on sperm vitality measured using

the eosin-nigrosin stain (fig. 5.2). The results revealed that incubating sperm with glucose at 30 and 50 mM promoted much greater sperm survival after 6 days of 43.4% (p<0.0001) and 51.4% (p<0.0001) compared with SPM samples at 7.1% survival.

Incubation of sperm with glucose did not result in a significant difference in CML levels between sperm incubated with glucose at either concentration and sperm incubated with SPM alone (fig. 5.1, p>0.05). Sperm motility was also measured over the course of the first 72 hours of incubation. At 72 hours, glucose supplementation had maintained sperm progressive motility at around 50% which was higher than sperm incubated with SPM, though not significantly so (p>0.05) (fig. 5.3). It is possible that the incubation period was

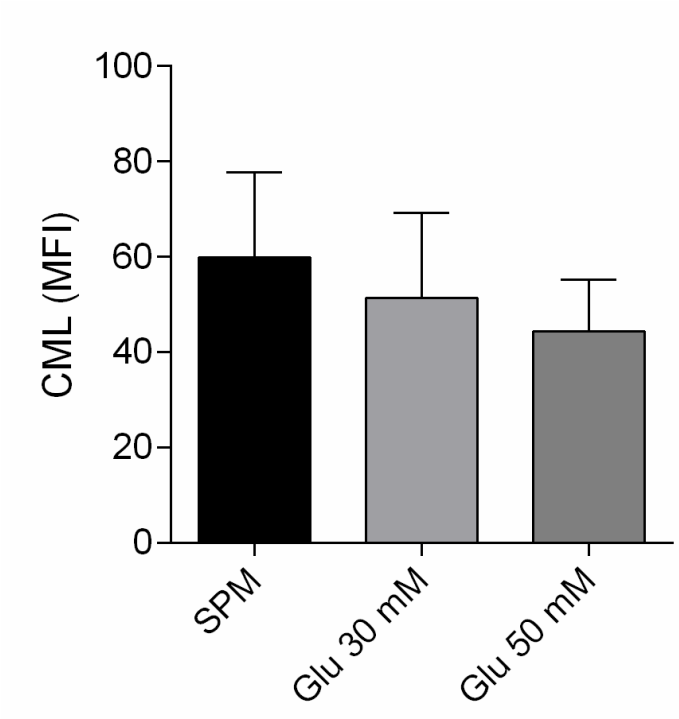


Figure 5.1 CML formation on sperm glycated with glucose for 6 days

Relative CML levels were measured as mean fluorescence intensity (MFI). CML levels in sperm incubated with glucose at 30 mM (n=4) and 50 mM (n=3) were not significantly different from sperm incubated with SPM alone (n=4) (p>0.05). Error bars represent the standard deviation of the mean.

not sufficient for glycation by glucose to take place, however it was unadvisable to extend the incubation period as the vitality of sperm incubated with the control media (SPM) would have reached 0%.

5.2.1.2 Fructose and mannitol do not glycate sperm *in vitro*

Since glycation of sperm with glucose was not evident in the time frame necessary, fructose was then tested as a potential physiological glycating agent. Fructose is a reducing sugar with the same molecular weight as glucose (180.16) and is the abundant sugar in seminal plasma. As a control, the non-reducing sugar mannitol was used. This sugar lacks the glycating

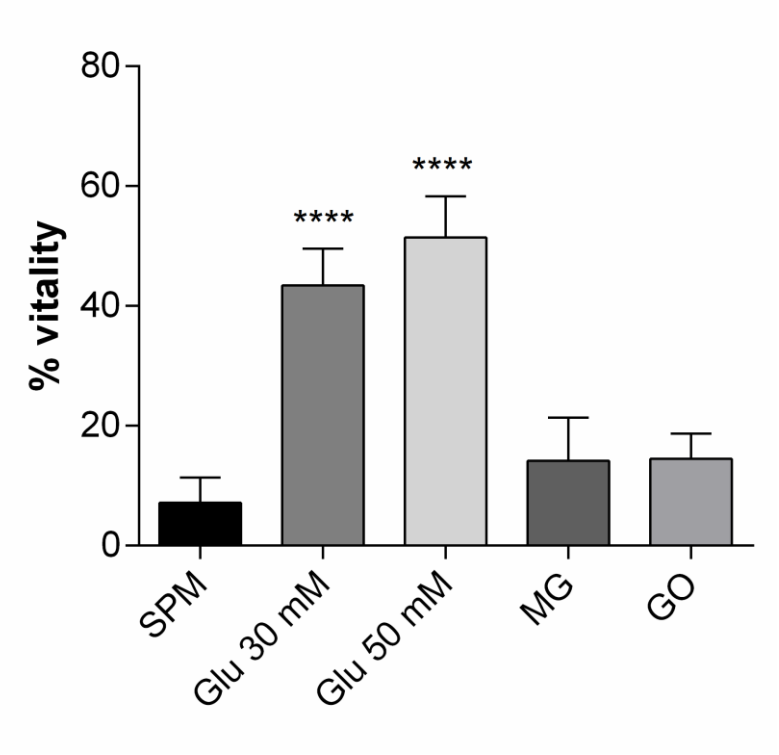


Figure 5.2 Vitality testing of glycated sperm incubated with glucose

Results show the % live sperm following glycation with glucose (n=7) (30 mM and 50 mM), MG (n=7) (50 µM) and GO (n=4) (50 µM) for 6 days, measured using the eosin-nigrosin stain. Sperm vitality decreased in all treatment groups during the incubation period but the presence of glucose significantly maintained vitality at a much higher level than SPM, MG and GO (**** = $p<0.0001$).

capacity of glucose and fructose yet also has a similar molecular weight (182.16). Neither fructose nor mannitol treatment resulted in a significant difference in CML levels compared to other treatments (p>0.05).

5.2.1.3 Toxicity testing of MG

As the reducing sugars were ineffective at glycating sperm over the 6 day time period, the reactive AGE intermediate, methylglyoxal (MG), was used. MG is a potent and rapid AGE precursor, reacting more readily with proteins than the aforementioned simple sugars. A kill curve was carried out over 48 hours and vitality measured with the eosin-nigrosin stain to determine the optimal concentration to use. Cell vitality was expected to consistently decrease over time, and as 48 hours was one third of the total incubation time of 6 days, a toxicity cut-off point was set at a decrease of one third of the original vitality. The concentration at which the number of live cells was reduced by less than one third was 50 µM (fig. 5.4), and this concentration was used for all further experiments with MG.

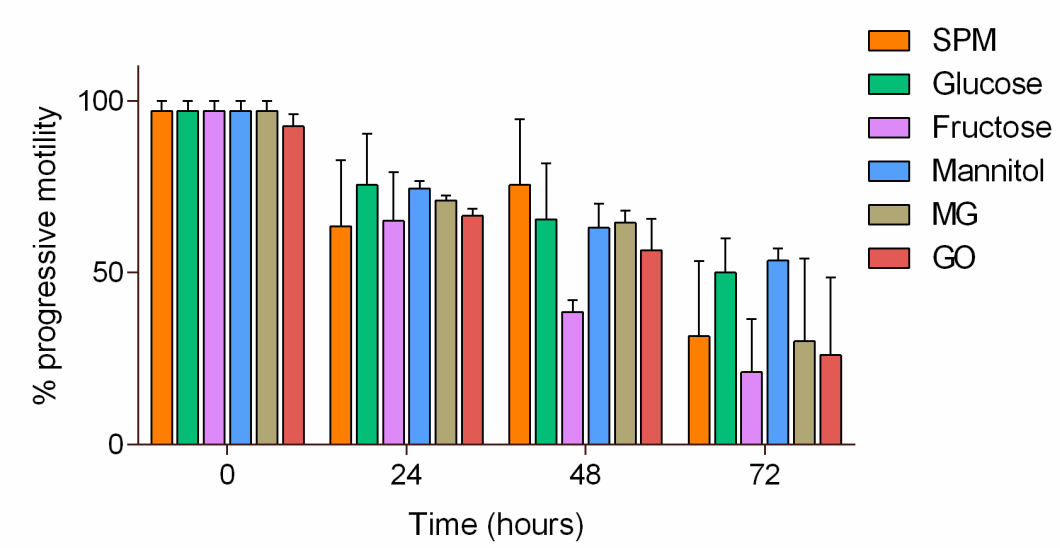


Figure 5.3 Effects of glycation on sperm motility

% Progressively motile sperm after 72 hours in culture with SPM, glucose, fructose, mannitol MG and GO (n=3). Two-way ANOVA with multiple comparisons showed that all treatments showed a significant decrease in sperm motility over time (p<0.05), however, none of the treatments were significantly different at 72 hours (p>0.05). Error bars represent the standard deviation of the mean.

MG treatment caused a substantial decrease in sperm vitality over 6 days to 14.1% and this was not significantly different from survival rate in SPM (p>0.05). This vitality data correlated with sperm motility data which showed that by the end of the incubation period, 100% of cells exposed to SPM and MG were immotile, while 30 mM and 50 mM glucose samples showed 31% and 41% total motility, respectively.

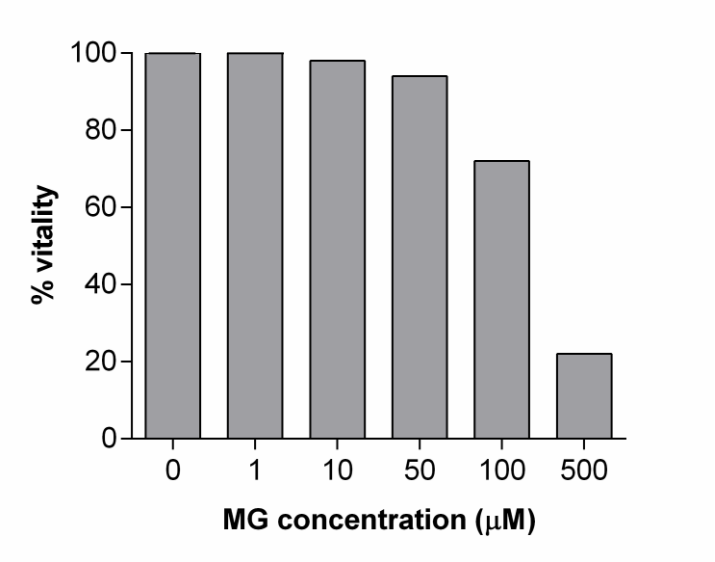


Figure 5.4 Methylgloxal kill curve

Grey bars show the % live sperm after 48 hours incubation with various concentrations of MG (0-500 μM). Vitality was determined by the eosin-nigrosin stain.

5.2.1.4 Glyoxal glycates sperm in vitro

Another reactive AGE intermediate - glyoxal (GO) - was included in the glycation experiments as it is the major intermediate source of CML, while MG primarily forms carboxy-ethyl-lysine (CEL), a structurally similar AGE. In glycation research these compounds are usually investigated simultaneously as they have a similar potency and prevalence in the body and are both implicated in AGE-related disease. Due to their structural similarity and

similar physiological concentrations, GO was used alongside MG in all experiments at 50 µM.

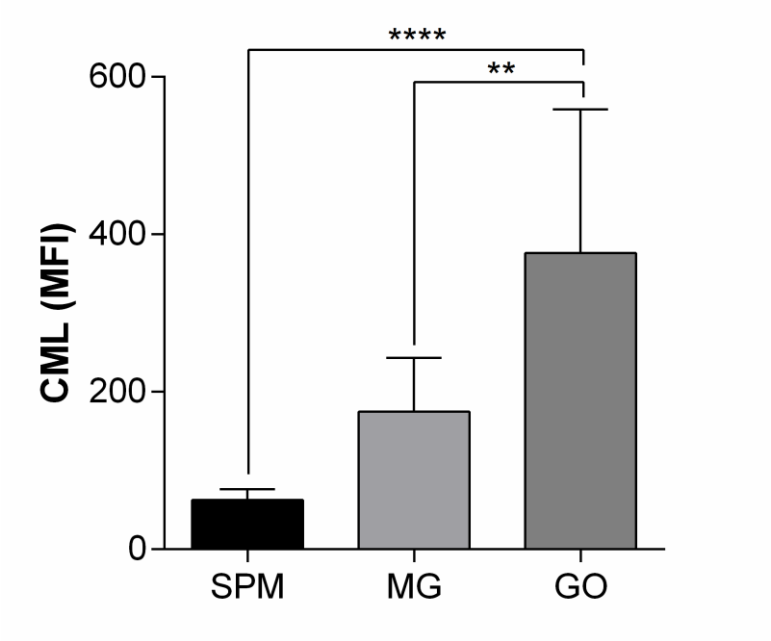


Figure 5.5 Glycation of sperm with MG and GO

CML formation on human sperm after a 6 day incubation with SPM (n=8) and with glycating agents, MG (n=8) and GO (n=5). Relative CML was measured as mean fluorescence intensity (MFI) of flow cytometry detection. One-way ANOVA analysis showed that GO caused significantly more CML formation than MG or SPM. Error bars represent the standard deviation of the mean. ****p<0.0001, **p<0.01.

Glycation of sperm with reactive AGE intermediates over 6 days resulted in higher levels of CML formation in sperm treated with MG and GO compared with SPM-treated sperm (fig. 5.5). However, a one-way ANOVA showed that only GO was significantly different from MG (p<0.01) and SPM (p<0.0001), while MG did not cause significantly higher levels of CML than SPM control (p=0.771). In terms of the effects of these factors on sperm motility, there

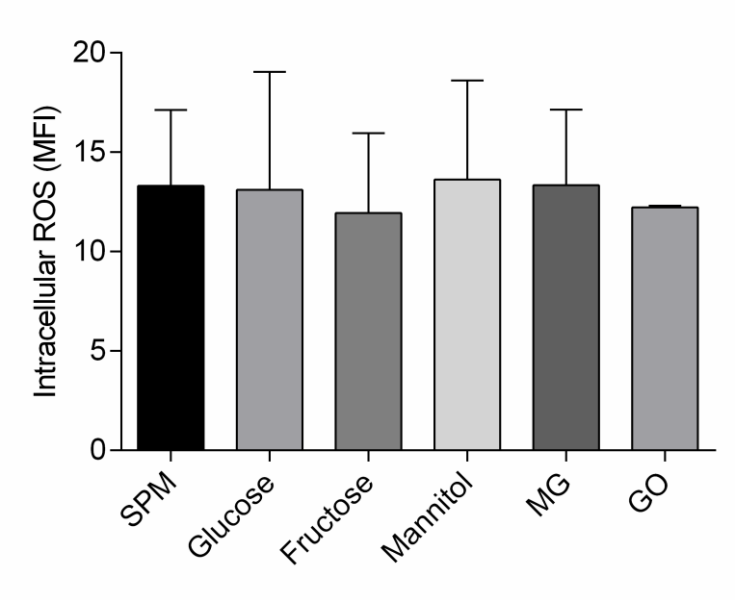


Figure 5.6 Intracellular ROS production in glycated sperm

Relative ROS levels in sperm treated with SPM (n=6), with the reducing sugars glucose (n=3) and fructose (n=3) (10 mM), a non-reducing sugar control mannitol (n=3) (10 mM), MG (n=6) or GO (n=3) (50 µM) for 6 days. ROS was measured as mean fluorescence intensity (MFI) of flow cytometry detection. One-way ANOVA analysis revealed no difference in ROS levels between any of the treatment groups. Error bars represent the standard deviation of the mean.

was a significant drop in sperm motility after 72 hours incubation, however motility was not significantly different to sperm incubated with SPM (p>0.05) (fig. 5.3).

5.2.1.5 Intracellular ROS production is not affected by glycating agents

Glycation of proteins and binding of AGEs to RAGE causes the release of reactive oxygen species (ROS). Intracellular ROS was therefore measured in sperm after the 6 day incubation period with sugars or intermediates to

identify if ROS generation was occurring. The results showed no significant difference in ROS levels between sperm from any of the treatment groups (p=0.992) (fig. 5.6). As the ROS levels were so low, a test was carried out with varying concentrations of MG (0-500 µM) to determine at what point high ROS production would occur (fig.5.7). There was a slight increase in ROS production from 50 µM onwards, but the clearest change was after the use of 500 µM MG, at which point ROS levels triple that of the preceding concentration.

5.2.1.6 Oxidative DNA damage is elevated in sperm treated with MG and GO

Due to the unstable nature of free radicals, ROS measurement can be unreliable and underestimate the level of damage caused to cells. Instead, the biological effects of damage by ROS were determined by measuring 8-oxoguanine levels as a marker of oxidative DNA damage. The results showed that 8-oxoguanine levels were significantly higher in sperm treated with GO than with MG (p=0.043) and SPM (p=0.018) (fig. 5.8). However, MG did not cause significantly more oxidative DNA damage than SPM (p=0.754). This trend mirrored the results of the glycation experiment in which GO caused an increase in CML levels.

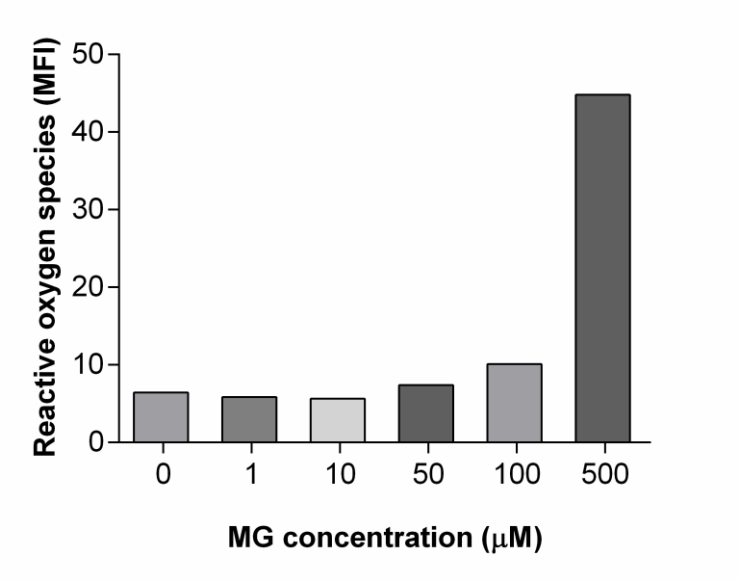


Figure 5.7 Intracellular ROS levels in sperm treated with MG

Relative ROS levels were measured in sperm treated with MG at various concentrations (0-500 µM) for 48 hours. ROS levels were drastically elevated only at the highest concentration of MG. ROS levels were measured as mean fluorescence intensity (MFI) during flow cytometry detection.

5.2.1.7 Immunocytochemistry for AGEs

Glycated sperm were stained by immunocytochemistry (ICC) for general AGEs and CML to identify the cellular locations of these compounds on the cells. Initial observations show AGE and CML staining on all anatomical regions of the sperm: the head, midpiece and tail. Corrected total cell fluorescence (CCF) was calculated to account for background levels of fluorescence in the images. This analysis was only carried out on the head regions of the sperm.

AGEs seem to show an even distribution across all parts of the sperm cell (fig. 5.9 B, E, H). At higher magnification (100X), the AGE staining on the sperm head shows a fine speckled pattern. This staining, as well as tail staining, showed higher reactivity in sperm treated with GO than with SPM or

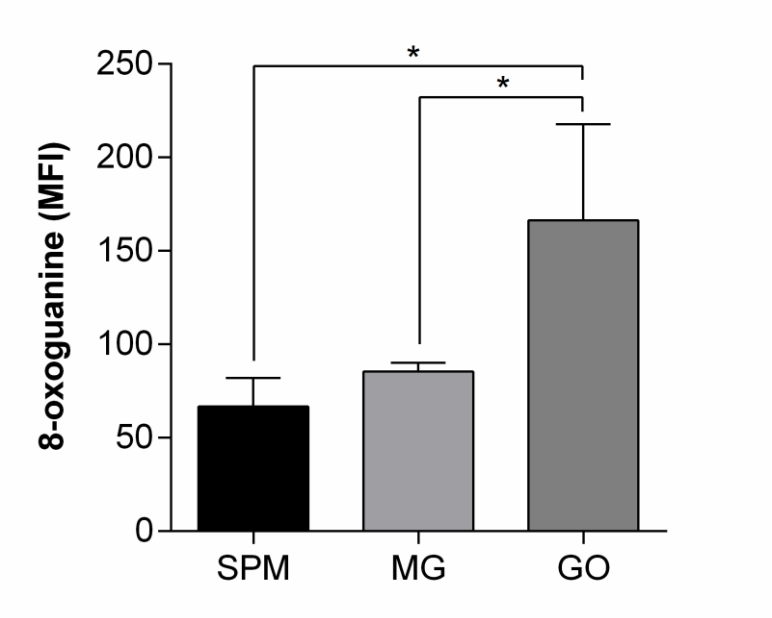


Figure 5.8 Oxidative DNA damage in glycated sperm

8-oxoguanine levels were measured in sperm treated with SPM (n=3) and reactive AGE intermediates, MG (n=3) and GO (n=3) (50 µM). 8-oxoguanine levels were determined as the mean fluorescence intensity (MFI) of flow cytometry detection. GO treatment caused significantly more 8-oxoguanine generation than SPM or MG (*p<0.05). Error bars represent the standard deviation of the mean.

MG. However, the CCF analysis showed that relative AGE intensity in the head region of GO-sperm was significantly higher than MG (p=0.0048) but not SPM (0.3232) (fig. 5.11). SPM-sperm also showed significantly higher AGE intensity than MG cells (p=0.044). The data suggest that AGE intensity in GO-sperm was not different from SPM-sperm, however it is clear from the images that tail intensity was very different between the treatment groups and should be accounted for alongside the CCF data.

CML fluorescence was faint in SPM-treated sperm, while showing a more intense staining in the tail region of the MG- and GO- treated cells (fig. 5.10 B, E, H). This was particularly prominent in the acrosomal head region in GO sperm, from the mid-section to the most anterior point (fig. 5.10 G). This is supported in the CCF data (fig. 5.12) which showed that GO cells had significantly higher CML staining than SPM (p=0.0419) and MG cells (p=0.0237), while CML in heads of MG-sperm was not different to SPM-sperm (p=0.999). These CCF data match that of the flow cytometry results for CML levels in sperm. As the CCF data does not account for tail intensity, it is possible that CML levels on tail regions in MG-sperm could be higher than that of SPM-sperm, as the images suggest (fig. 5.10 B and E).

Negative controls for AGEs (fig. 5.9 J-L) and CML (fig. 5.10 J-L) showed a slight amount of autofluorescence or non-specific binding, but these levels were minimal and unlikely to compromise the observations.

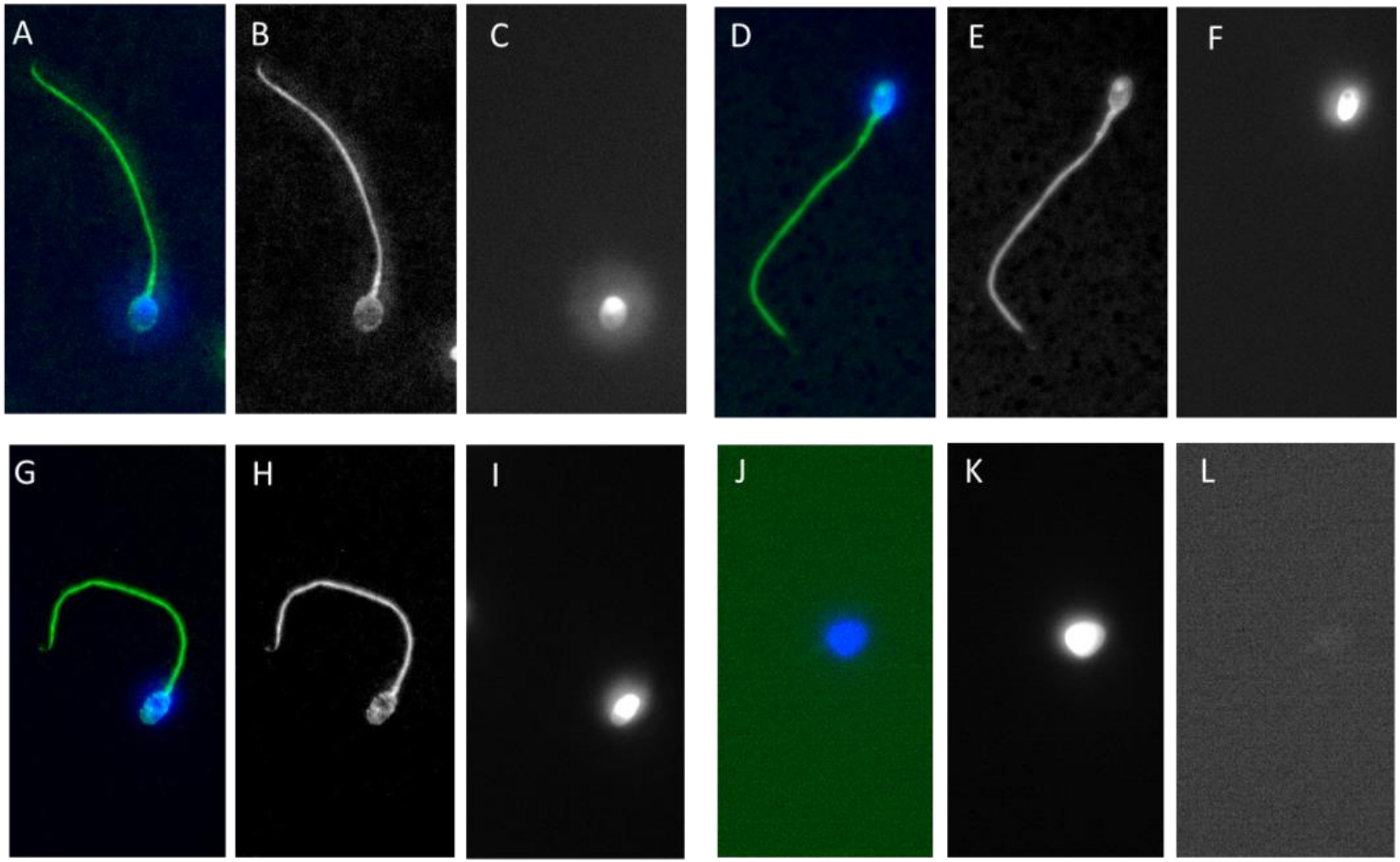


Figure 5.9 Immunocytochemistry for the detection of general AGEs on sperm. ICC was performed on sperm incubated with SPM (A-C), MG (D-F) and GO (G-I) for 6 days. Nuclear staining with DAPI is shown in the middle column, AGE positivity measured in the FITC channel is shown in the right hand column, and merged images are shown in the left-hand column. All treatment groups showed positive staining for AGEs in the head and tail regions. In MG and GO groups, there was more intense staining, particularly on the tail region in the MG and GO groups. Overall, GO had the most intense head staining. Magnification at 100x oil immersion.

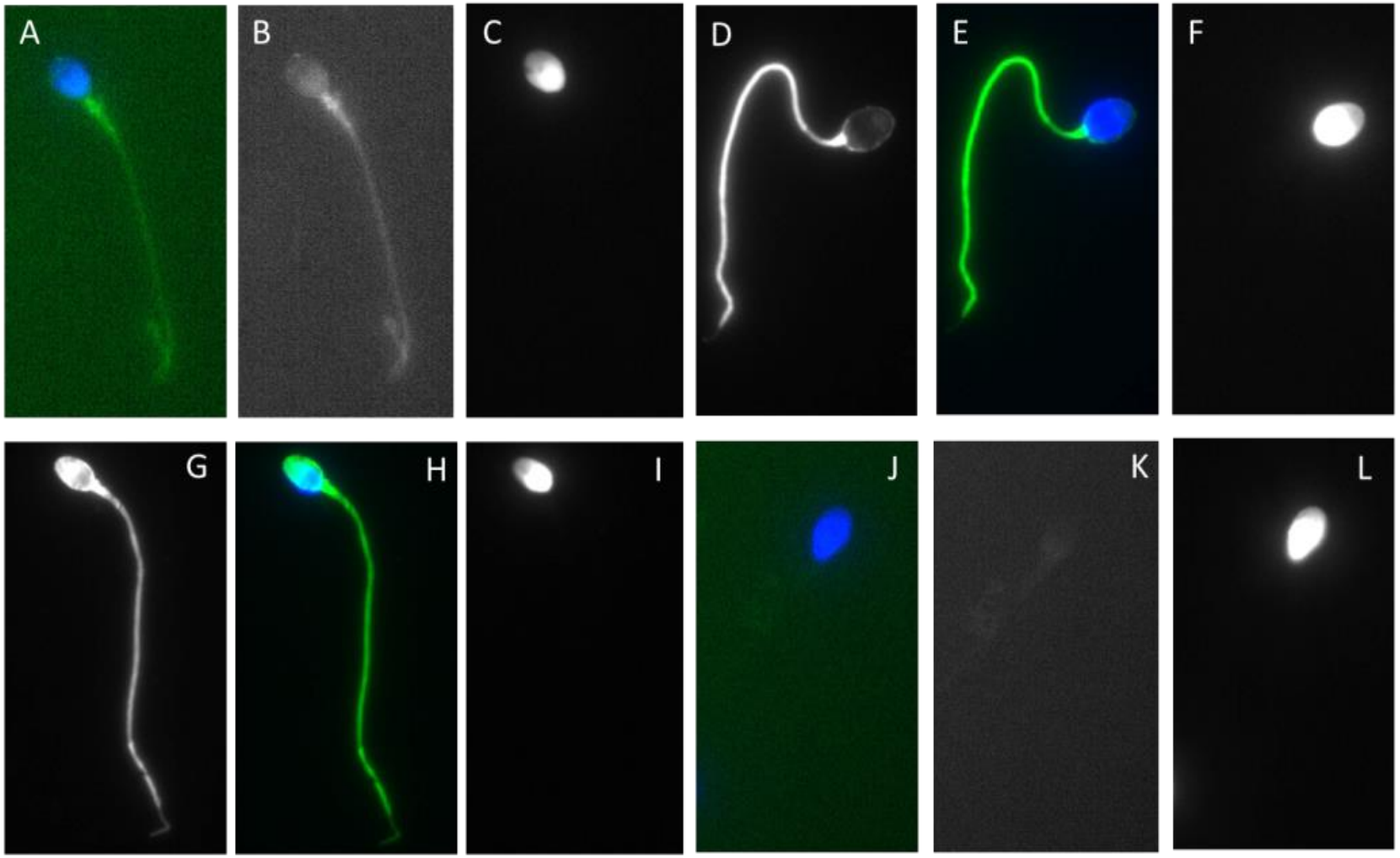


Figure 5.10 Immunocytochemistry for the detection of CML on sperm. ICC was performed on sperm incubated with SPM (A-C), MG (D-F) and GO (G-I) for 6 days. CML was not as abundant on sperm treated with SPM as it was on MG- and GO-treated sperm, and this difference is clear from the images and is supported by cell intensity quantification. Magnification at 100x oil immersion.

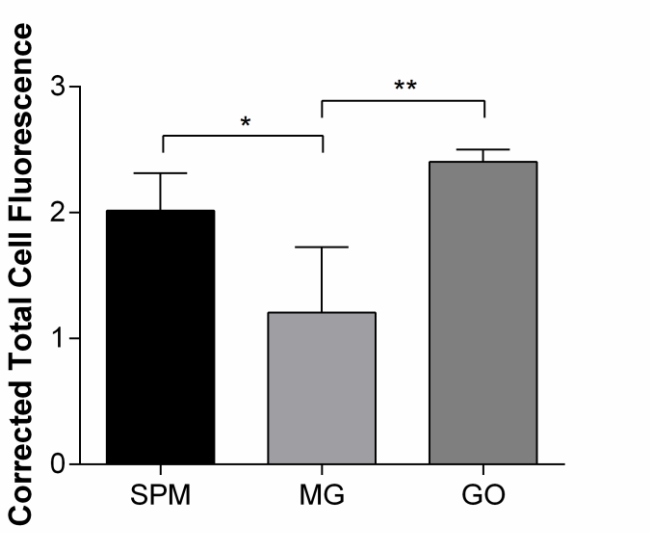


Figure 5.11 Corrected Total Cell Fluorescence for AGE ICC

Fluorescence intensity of general AGEs in sperm treated with SPM, MG and GO for 6 days (n=3). Corrected total cell fluorescence (CCF) represents fluorescence of sperm heads only. GO showed higher CCF than MG and SPM showed higher CCF than MG. Error bars represent standard deviation of the mean. *p<0.05, **p<0.01.

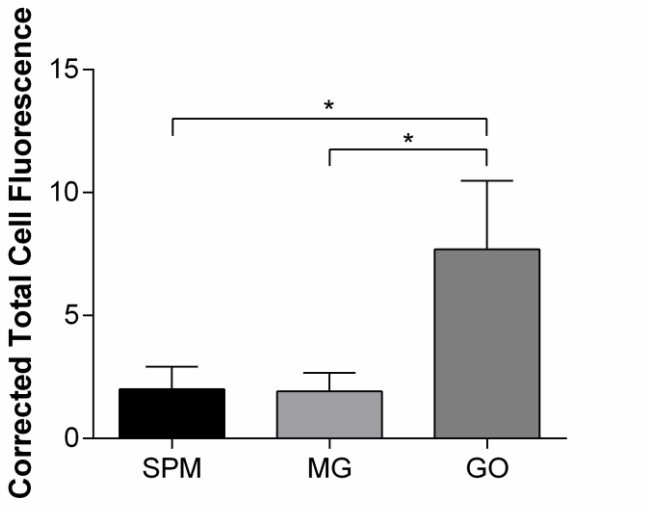


Figure 5.12 Corrected Total Cell Fluorescence for CML ICC

Fluorescence intensity of CML in sperm treated with SPM, MG and GO for 6 days (n=3). Corrected total cell fluorescence (CCF) represents fluorescence of sperm heads only. GO treatment showed significantly higher CML reactivity in sperm heads than SPM and MG treatment. Error bars represent standard deviation of the mean *p<0.05.

5.2.2 Short term glycation of sperm

5.2.2.1 Glyoxal glycates sperm in 2 hours

As the level of glycation was extremely high in sperm treated with GO after 6 days, the same glycation experiment was carried out over a 4 hour time period, with CML measurements taken at 2 hour intervals. When comparing time points 0, 2 and 4 hours, a two-way ANOVA with Tukey's test for multiple comparisons showed a significant increase in CML levels in GO-treated cells from 0 to 2 hours (p<0.0001), 0 to 4 hours (p<0.0001) and 2 to 4 hours (p<0.01) (fig. 5.13). However, there was no significant change with either SPM or MG across any of the time points (p>0.05). The levels of CML in GO-treated cells was significantly higher than that of MG- and SPM-treated cells at 2 hours (p<0.0001) and 4 hours (p<0.0001). MG treatment was not significantly different to SPM treatment at any time (p>0.05).

When CML values were compared as the percentage difference from the initial time point (0 hours), GO treatment showed a 225% increase after 2 hours which increased to 259% at 4 hours and a 500% increase after 6 days. MG treatment showed a non-significant 37% increase after 2 hours and this did not change up to 4 hours, and there was a 179% increase in MG levels after 6 days, but this was not significantly different to SPM treated cells.

5.2.2.2 Sperm motility is not affected by short term glycation

To determine whether the increase in glycation seen in sperm is associated with a change in sperm motility, the percentage of progressively motile sperm was simultaneously measured at each time point when removing cells for CML analysis. The results showed no significant difference in sperm motility across the 4 hours of incubation with SPM, MG or GO treatments (fig. 5.14).

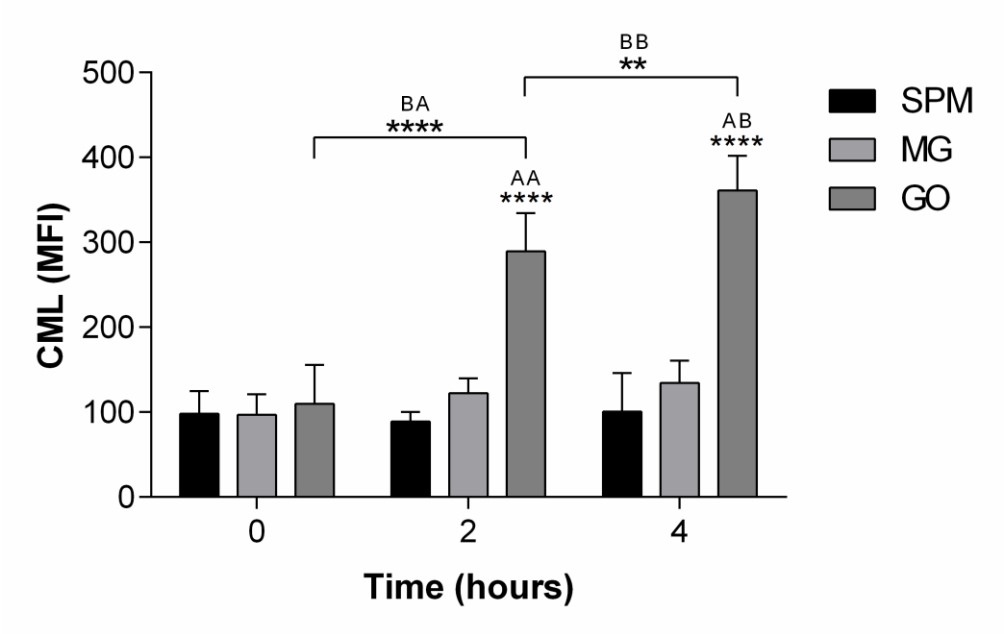


Figure 5.13 Short-term glycation of sperm

CML levels in sperm treated with SPM, MG and GO for 4 hours (n=3). CML was measured as the mean fluorescence intensity (MFI) of flow cytometry fluorescein detection. Sperm incubated with GO showed an increase in CML at 2 hours (BA) and 4 hours (BB), and this was significantly higher than MG and GO at these time points (AA and AB). Error bars represent standard deviation of the mean. ****p<0.0001, **p<0.01.

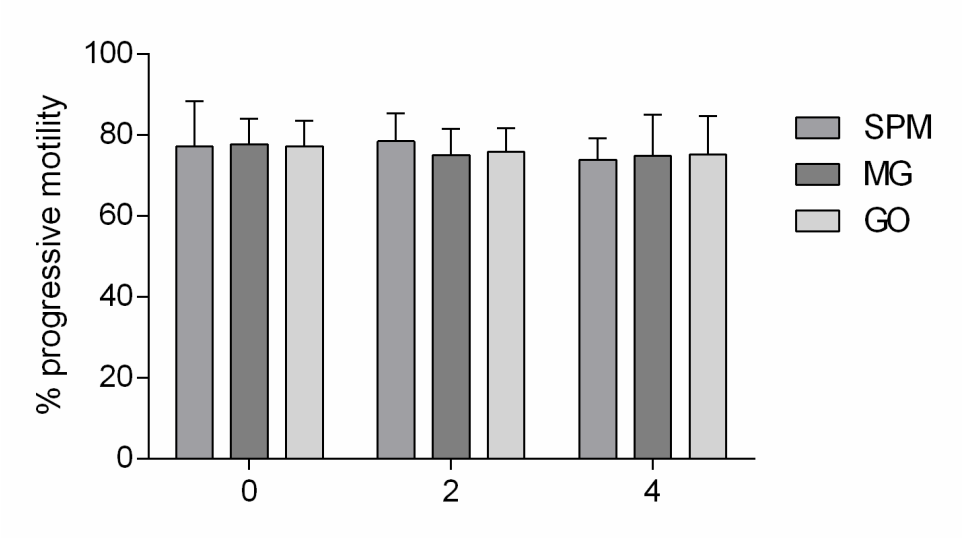


Figure 5.14 Effects of short-term glycation on sperm motility

Sperm progressive motility during 4 hour glycation with SPM, MG and GO (n=3). Two-way ANOVA was performed to identify differences between treatment and time. There was no significant difference in progressive motility over 4 hours. Error bars represent standard deviation of the mean.

5.2.3 Sperm glycation in obese men

5.2.3.1 Semen parameters are not different between obese and normal weight men

To determine whether there is an elevation in sperm AGEs in obese men, CML levels were compared between two groups of obese and normal weight men. The mean BMI of the obese group was significantly higher than the control group at 34.34±3.88 kg/m^2 compared to 23.28±1.31 kg/m^2 (p=0.001). The semen parameters for both groups are shown in table 5.1. The mean sperm concentration, semen volume, % progressive motility and % total motility were all lower in the obese group, however this was not significant (p>0.05). Similarly, the % immotile cells was higher in the obese group but again this was not significant (p>0.05). The mean age for the obese group was 48.4 years (±4.88) while the mean age for the control group was 29.2 years (±5.12) (p=0.0003). Abstinence time was between 2 and 5 days for all cases.

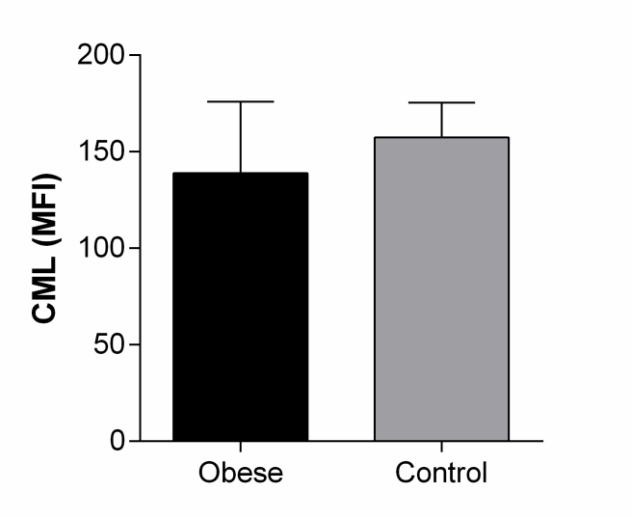


Figure 5.16 CML levels in the sperm of obese and non-obese men

CML was detected using an anti-CML fluorescent antibody and the Mean Fluorescence Intensity (MFI) was recorded by flow cytometry by fluorescein detection. There was no significant difference in sperm CML levels between obese men (n=5) and normal weight men (n=5). Error bars represent standard deviation of the mean.

5.2.3.2 CML levels are not elevated in the sperm of obese men

CML levels were measured in the prepared sperm of obese and control men by flow cytometry using the same method as for sperm that underwent *in vitro* glycation. There was no significant different in CML levels between the obese and control groups (p=0.344) (fig. 5.16).

Table 5.1 Semen parameters of obese and non-obese men

Semen parameters	Obese (n=5)	Control (n=5)	*p*-value
Concentration (x10^6/ml)	35.97±28.48	62.6±18.1	0.116
Volume (ml)	2.7±2.12	4.7±1.2	0.157
Progressive motility (%)	45±22.49	58.06±7.24	0.252
Total motility (%)	54.8±24.23	70.12±8.28	0.218
Immotile (%)	45.2±24.24	29.88±3.61	0.218

Means and SD are shown for each semen parameter in the obese (n=5) and control (n=5) groups. Independent t-tests for differences between the groups were carried out and *p*-values are shown. Semen parameters were not statistically significantly different between the groups (p>0.05).

5.2.4 Effects of glycation on sperm hyaluronidase

Increased AGE production has been associated with male infertility (Mallidis, *et al* 2009). This study has demonstrated that glycation does not affect sperm motility. However, as AGEs are important instigators of oxidative stress and cell dysfunction in numerous diabetic complications, lifestyle exposures and aging, we tested if these compounds would have an effect on sperm at a molecular level by perturbing hyaluronidase function. We chose to investigate hyaluronan binding capacity of sperm as this is an essential process for successful fertilization. To investigate the effects of glycation on

hyaluronan binding, the hyaluronan binding assay (HBA) was therefore carried out on glycated sperm over 4 hours to determine whether there was a change in capacity for sperm to bind to HA. In addition to the cell-based assay, we also glycated recombinant hyaluronidase *in vitro* and used a hyaluonidase-substrate gel assay to determine enzymatic activity. Molecular cloning was first attempted to generate the recombinant protein, however this was unsuccessful. As an alternative, a commercial hyaluronidase enzyme, Cumulase™, was used for the *in vitro* glycation.

5.2.4.1 Hyaluronan binding capacity is not affected by short term glycation

The HBA assay showed that sperm showed no significant changes in binding to HA following incubation with MG or GO (fig. 5.17). Hyaluronan binding did not drop below 90% in any of the treatment groups, showing that the glycation seen in 4 hours by GO was most likely not affecting the function of the cell surface hyaluronidase protein.

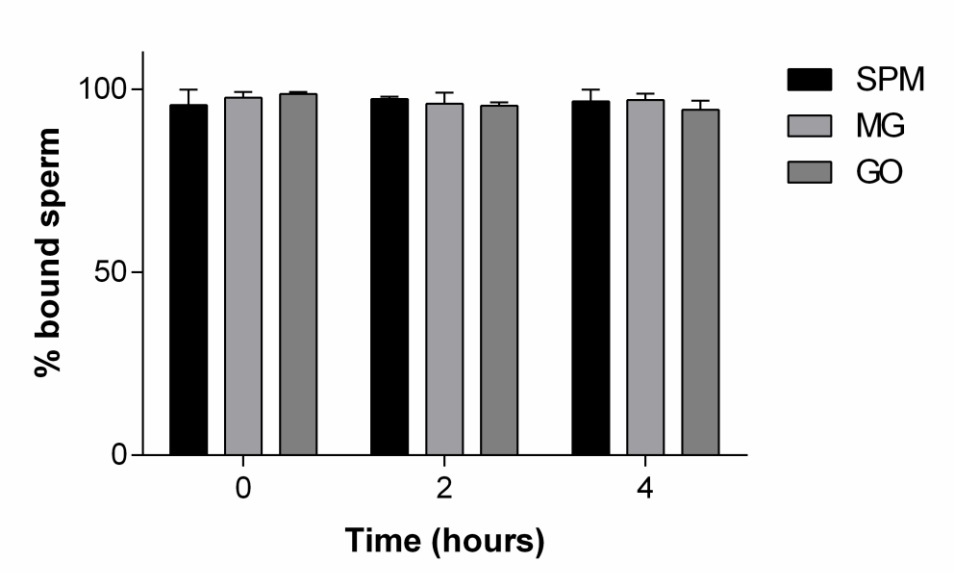


Figure 5.17 Hyaluronan binding assay for glycated sperm

HA binding of sperm incubated with MG and GO over 4 hours (n=3). No difference was seen in hyaluronan binding capacity at 2 or 4 hours. Sperm maintained a high level of binding throughout. Error bars represent the standard deviation of the mean.

5.2.4.2 Recombinant SPAM1 is insoluble when expressed in E.coli

Numerous extraction methods were tested for the purification of cloned SPAM1 protein, however it remained insoluble and could be seen in the insoluble pellet following each extraction (fig. 5.18 lane 6). The protein was successfully expressed in E.coli when the cells were induced (fig. 5.18, arrow), however, the protein could not be seen in the supernatants of the extraction buffers (wells 3, 4 and 5) whereas a large band was seen in the pellet. The SPAM1 transcript product was 58 kDa and the GST tag is approximately 26 kDa, making the total weight around 84 kDa, which can be seen as the band above the 75 kDa molecular weight mark.

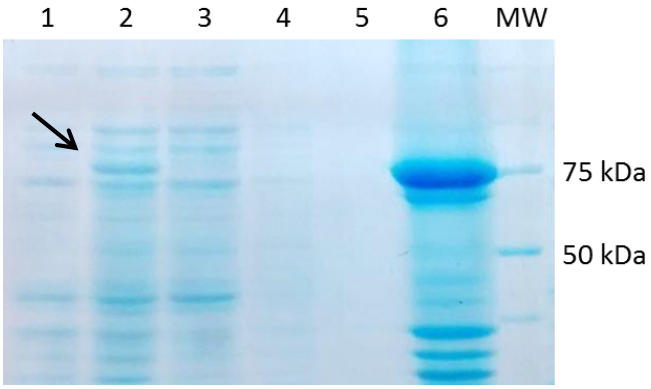


Figure 5.18 Recombinant SPAM1

SPAM1 was expressed in E.coli and extracted with lysozyme, sodium deoxycholate, lysis buffer 1 and 2 and extracted in solubilisation buffers 1 and 2. Wells represent 1) E.coli cells not induced, 2) E.coli induced with 0.2 mM IPTG, 3) 1st supernatant, 4) 2nd supernatant, 5) 3rd supernatant, 6) cell pellet. All SPAM1 transcript was present in the insoluble protein pellet.

5.2.4.3 Hyaluronidase substrate gel assay development

In order to determine the effect of glycation on hyaluronidase function, a substrate gel assay was developed in which a polyacrylamide gel was embedded with HA, which was then degraded by the hyaluronidase enzyme that was electrophoresed onto the gel. The gel is then stained for undegraded HA: the gel stains blue for HA while degraded zones remain unstained, resulting in a negative image. A second stain with coomassie blue reveals the remaining electrophoresed protein that is left in the gel, visualised as a dark band (fig. 5.19). The optimal incubation buffer composition and pH for observing hyaluronidase activity in the Cumulase enzyme was determined. All tests using sodium acetate buffer showed some degradation of HA in the gels (fig. 5.19), however incubation buffers at pH 4

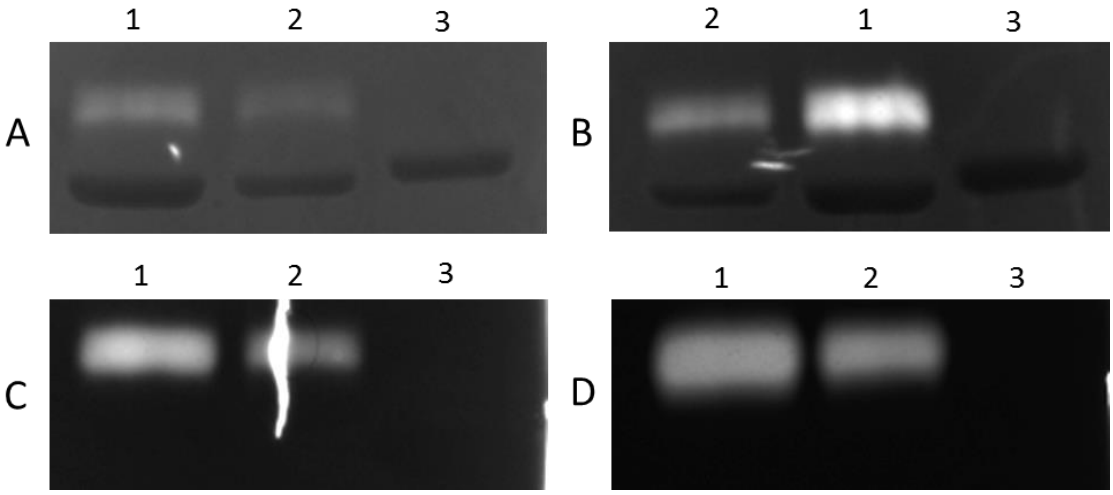


Figure 5.19 Hyaluronidase substrate gel assay development

Measurement of Cumulase activity in a substrate gel assay using enzyme activation buffer at pH 3 (A), pH 4 (B), pH 6 (C) and pH 7 (D). Two concentrations of Cumulase were used: 0.25 mg/ml (well 1) and 0.125 mg/ml (well 2). BSA was also tested at 0.25 mg/ml (well 3). White bands represent areas of the gel where HA has been degraded due to hyaluronidase activity. A higher concentration of Cumulase caused greater degradation of HA in the gel, observable as larger and brighter bands, while BSA showed no degradation of HA. pH 4 (B) and pH 7 (D) showed the clearest bands in this experiment.

and pH 7 were the most optimal (fig. 5.19 B and 1D). The Cumulase enzyme contains HSA (66.5 kDa) as a cofactor and a control was run for this containing BSA (66.5 kDa) at 0.25 mg/ml (fig. 5.19 A-D, well 3). The protein bands in the Cumulase wells (1 and 2) showed the same sized protein band as the BSA control well. Therefore the protein band visualised most likely corresponds to the cofactor, rather than the hyaluronidase. Although the gels incubated at pH 7 showed high levels of enzyme reactivity and clear regions of HA degradation, they did not stain well for protein (fig. 5.19 D). This is in comparison with pH 4 where the protein bands could be seen clearly (fig. 5.19 B). Different concentrations of Cumulase were used in this assay development (0.25 mg/ml and 0.125 mg/ml) and can be identified by the size of the dark protein bands (fig 5.18 A and B).

5.2.4.4 Glycation of Cumulase does not affect enzyme function

The conditions used for the hyaluronidase substrate gel assay, based on the assay development, were incubation in sodium acetate buffer at pH 4 for 16 hours. Cumulase which had been glycated with glucose, fructose, mannitol, MG or GO for 0, 3, 7 or 14 days were electrophoresed onto the gel and their HA degradation capacity was observed. The results showed that HA degradation by Cumulase, measured by the size of degraded HA sites, was not different following incubation with sugars and AGE intermediates (fig. 5.19). The enzyme retained a similar level of activity at 14 days as at start of the experiment. Unlike during the assay development, the protein band which was visualised as a dark band, was not present. Instead, a clear band appeared in the same position but was absent from the control BSA well. This could indicate HA degradation, or alternatively the protein present in the gel could be preventing penetration of the dye, however this is unlikely as the BSA sample did not reveal this same white band (fig. 5.19, well 7).

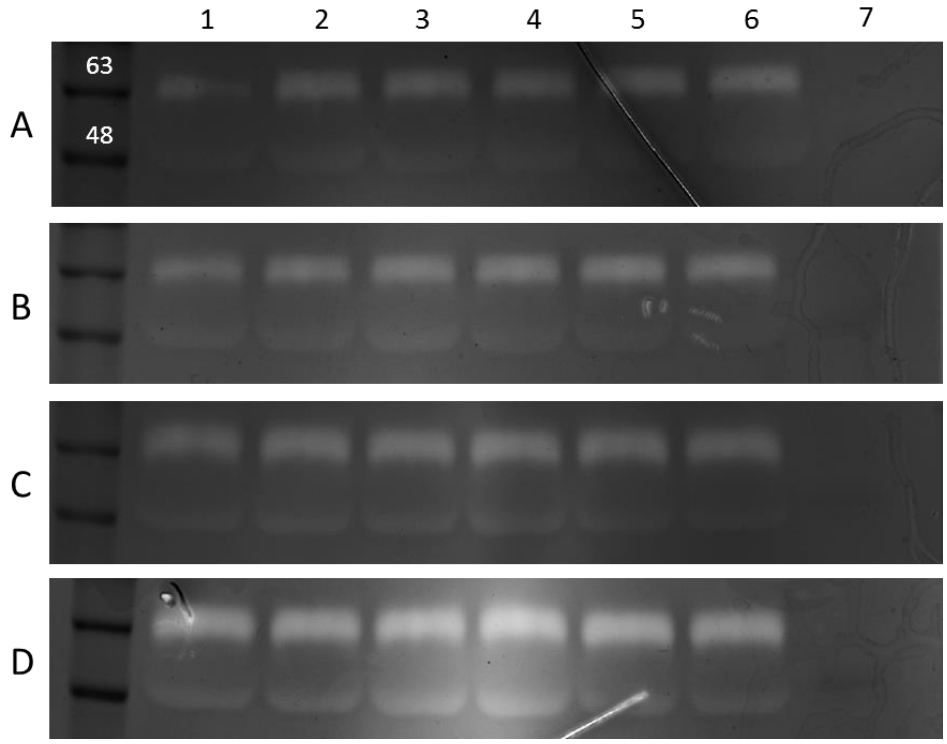


Figure 5.20 Cumulase activity measured using a hyaluronidase substrate gel assay

Cumulase was incubated with 1) PBS; 2) glucose; 3) fructose; 4) mannitol; 5) MG and 6) GO over 0, 3, 7 and 14 days (A-D, respectfully) to determine the changes that could occur in this enzyme as a result of glycating conditions. There was no observable difference in band size between the treatment groups (1-6) indicating no change in hyalurondiase activity. BSA alone (7) did not cause any HA degradation. Two bands appear around the 63 kDa and 48 kDa molecular weight markers. All gels were run on the same day.

5.3 Discussion

Glycation is the spontaneous non-enzymatic reaction of a carbonyl group of a reducing sugar with a protein amine group, which through multiple successive reactions forms covalent irreversible AGEs. AGE-modification causes damage to biomolecules and modifies their function as well as causing cellular stress through the generation of ROS and become elevated with age and in disease. As AGEs have been located on sperm cells and in the male reproductive tract, this study sought to use an *in vitro* experimental model to show the mechanism by which sperm is glycated by physiological compounds. The reactive AGE intermediates, MG and GO are potent glycating agents, around 20,000 times more reactive than glucose (Thornalley, 1996), and are physiologically elevated in disease states. These intermediates were therefore used as an accelerated model for glycation. The main findings of this study were that CML, a common and potent AGE, was rapidly formed on sperm when incubated with the AGE intermediate GO. Glycated sperm experienced higher levels of oxidative DNA damage, indicating a role for oxidative stress in AGE-mediated cellular damage.

Over a 6 day period in culture, CML levels did not increase in sperm incubated with glucose compared with SPM alone. This was unsurprising as most studies investigating the chemical processes of glucose-mediated glycation have shown that this can take from 1 to 4 weeks, often as long as the turnover time of the proteins *in vivo* (Coussons *et al.,* 1997; Kislinger *et al.,* 2003). This low reactivity is logical as being the most abundant sugar within biological systems, glucose reactivity would cause immense damage to biomolecules. Glycation is directly dependent on glucose concentration and time (Kislinger *et al.,* 2003) but due to the limited time that sperm can be maintained in culture, the concentration of glucose was used at concentrations (30 mM and 50 mM) higher than physiological levels (5 mM in blood; 1 mM in seminal plasma). However, neither 30 mM nor 50 mM glucose had an effect on CML levels. Although this end-stage AGE had not been formed, early-stage products of the Maillard reaction such as fructose-lysine may have been generated. In fertility clinics, sperm are maintained in

sperm preparation media for a number of hours. This media is optimised for sperm function and contains glucose at approximate levels of less than 0.1% (Origio, 2015), which the results of this study suggest would be insufficient for glycation-mediated damage to occur to sperm.

Another reason for the lack of CML formation could be in part due to sperm metabolising the glucose in solution, causing the concentration to decrease to levels insufficient for glycation. In support of this, the vitality test showed that the presence of glucose promoted sperm survival in comparison to SPM, MG and GO, and furthermore, sperm progressive motility was maintained at a high level following three days of incubation in comparison to other compounds. This suggests that sperm are actively utilising the glucose as a source for ATP generation through glycolysis as well as using oxidative phosphorylation for ATP generation in the mitochondria. Both these mechanisms for ATP generation are thought to be relevant to sperm function (reviewed in du Plessis *et al.,* 2015). Although replenishment of glucose in the media would have increased the rate of glycation, this is likely to have been negligible in this time frame.

Unlike the stable ring carbonyl structure of glucose, fructose exists more abundantly in the open-chain aldose form (Bunn and Higgins, 1981) and can form AGEs up to 10 times more readily than glucose, resulting in fluorescent and cross-linking products (Suárez *et al.,* 1989; Takeuchi *et al.,* 2010). However, in this study fructose (10 mM) did not show higher CML levels than SPM or glucose when incubated with sperm. Fructose is secreted from the seminal vesicles and is another energy source for sperm in the ejaculate to drive motility, however sperm only come into contact with seminal plasma upon ejaculation, in which case there would be no window of time for glycation to occur.

The AGE intermediates GO, MG and 3-deoxyglucosone (3DG) are reactive carbonyl compounds (RCC). They are formed during the Maillard reaction but also from the oxidation of AGEs (Thornalley *et al.,* 1999). RCCs rapidly form Schiff's bases with amino acids, hence their greater reactivity than glucose, and eventually form imidazolone adducts with molecules. This can

cause cytotoxicity to different cell types (Suzuki *et al.,* 2010). This study showed that exposing sperm to GO for as little as 2 hours caused a significant increase in CML levels compared with SPM and MG. This semi-quantitative method of analysis showed that CML levels were 225% higher after 2 hours, 259% higher after 4 hours and a 500% higher after 6 days in culture. Therefore this *in vitro* model has resulted in extremely rapid glycation by this intermediate compound. *In vivo* concentrations of MG and GO are approximately 1-5 µM and 0.1-1 µM intracellularly and 100-120 nM in blood plasma (Thornalley *et al.,* 1999). Although the *in vitro* concentrations used in this study were much higher than physiological levels, this was necessary in order to illustrate the potential effects of these compounds at a measurable level. Furthermore, the concentrations were lower than those used in *in vitro* cell assays elsewhere (Cantero *et al.,* 2007). The major AGE product of MG is carboxyethyllysine (CEL), and isomer of CML. This may explain the small increase in CML levels in MG-treated sperm as some antibodies against these compounds show cross-reactivity (Koito *et al.,* 2004). To further this investigation, CEL could be measured to determine the full effects of MG, as well measurement of the glyoxal-lysine-dimer (GOLD) and methylglyoxal-lysine-dimer (MOLD) would reveal whether glycated molecules were undergoing cross-linking, one of the damaging features of glycation (Odani *et al.,* 1998).

Immunocytochemical staining of glycated sperm was carried out in order to demonstrate the distribution of general AGEs and CML on the sperm cells. The staining of AGEs and CML along the entire length of the cell, including head, midpiece and tail is in accordance with that staining pattern found by Mallidis *et al.* (2009). The paper also describes high immunoreactivity to CML on the head acrosomal cap region in diabetics, compared to non-diabetics where immunoreactivity was low, and this is similar to the higher level of CML staining found here on sperm glycated with GO compared with SPM. The use of this *in vitro* assay may therefore be a good model for mimicking the diabetic environment and shows that this region may be particularly vulnerable to glycation in diabetic and ageing men.

AGEs and AGE intermediates have been found to increase with advancing age and in obese individuals (Uribbari *et al.,* 2007; Uribbari *et al.,* 2015) and sperm quality has also been shown to be reduced in these groups (Begueria *et al.,* 2014; Hammoud *et al.,* 2008). To investigate whether obesity might also lead to elevated sperm AGEs, CML was measured in obese men. No significant difference was found in CML or semen parameters between the groups, despite sperm concentration, motility and volume being lower in the obese group. One explanation for there being no difference in CML levels may be that prepared sperm was used for the flow cytometry analysis, which was separated by density gradient centrifugation before CML measurement. This was for the purpose of removing non-sperm cells and debris, however it would also have removed non-motile or immature sperm from the samples which could be the cells with the highest levels of AGEs. Mallidis *et al.,* (2009) located CML throughout the cells of the seminiferous epithelium, and particularly in the nuclei of spermatogonia and spermatocytes, while less so in the nuclei of mature spermatids. Therefore, the reason for no difference being seen in CML between obese and normal weight men may be because of the removal of the CML-positive population of immature sperm. The finding of no difference in semen parameters between the obese and non-obese group has been shown elsewhere (Eisenberg *et al.,* 2014), but the low numbers of participants used in this small study mean that there is insufficient evidence to draw conclusions from.

RCCs cause rapid changes to proteins: GO and MG have been shown to cause rapid generation of CML on proteins such as BSA and lysozyme *in vitro* (Glomb and Monnier *et al.,* 1995; Millar *et al.,* 2002). Therefore the CML production seen in the first four hours on sperm is probably occurring on structurally available proteins such as receptors on the sperm membrane, while later CML generation is occurring as a result of adduct formation on intracellular proteins and DNA. Although AGE formation was originally thought to only effect stable extracellular proteins, such as collagen, research now shows that glycation adducts can quickly occur on intracellular molecules such as actin (Boucher *et al.,* 2015) and on DNA (Wang *et al.,* 2010).

Cell-based *in vitro* glycation assays have previously shown that cell receptors such as the platelet derived growth factor receptor are vulnerable to glycation (Cantero *et al.,* 2007). Critically, modification of these receptors by AGE intermediates can alter their function, as was shown through inhibited epidermal growth factor receptor signalling (Portero-Oterin *et al.,* 2002). Sperm participate in a number of receptor-ligand interactions to which glycation could be detrimental, such as for the hyaluronidase activity of *Spam1*, which is responsible for degradation of HA oligosaccharide chains found in the cumulus layer of granulosa cells surrounding the oocyte (Evison *et al.,* 2009). Additionally, as *Spam1* is located on the acrosomal membrane where a high level of CML immunoreactivity was observed and as *Spam1* contains a number of lysine and arginine residues, this enzyme could be affected by glycation. Therefore, the function of cellular and recombinant *Spam1* protein was investigated in response to glycation.

Cellular hyaluronidase activity was analysed using a HA binding assay (HBA), in which sperm bind to HA-coated plates, and is often used as a measure of sperm quality though not routinely in fertility clinics. Sperm binding to HA was not affected following short-term glycation and this may be because the CML levels seen at this stage are affecting proteins other than *Spam1.* As the HBA requires motile sperm in order to distinguish between sperm that are bound (hyaluronidase active) and unbound (hyaluronidase inactive), the experiment could not be carried out at 6 days when sperm motility is mostly zero.

As an alternative, the effects of glycation on recombinant *Spam1* were investigated for up to 14 days. Other *in vitro* protein glycation models have shown significant effects on protein structure and function in response to AGE intermediates, such as with histones (Ashraf *et al.,* 2015) and insulin (Jia *et al,* 2006). A hyaluronidase substrate gel assay was developed to measure the changes in hyaluronidase activity and this was successful in the first stages. The assay was based on two previous studies (Mio *et al.,* 2001; Zhu *et al.,* 1994) and the optimal conditions used for subsequent analysis of activity of a commercial hyaluronidase enzyme, Cumulase, were incubation of gels in sodium acetate buffer (pH 4) for 16 hours. As the use of

recombinant protein allowed the assay duration to be extended, the simple reducing sugars glucose and fructose and the non-reducing sugar mannitol were investigated alongside MG and GO. No difference was observed in the hyaluronidase activity between Cumulase incubated with any of these compounds and SPM alone. In addition, the enzyme maintained the same level of activity after 14 days as at the beginning of the assay. This therefore likely concludes that the CML compounds located on sperm in this study and elsewhere (Mallidis *et al.,* 2009) were not affecting the membrane-bound *Spam1* protein.

Sabeur *et al.* (1997) previously reported two versions of *Spam1*. The first 64 kDa protein is GPI-anchored to the sperm plasma membrane and has a role in initial degradation of HA in the cumulus layer surrounding the oocyte and is functional at neutral pH (Lin *et al.,* 1994). Following binding to the ZP, the acrosome reaction is initiated and a soluble cleaved version of *Spam1* is released which has acidic function and is responsible for further HA degradation following ZP binding. In this assay, the full length *Spam1* (64 kDa) was observed to have activity at both pH 7 and pH 4. In the Cumulase assay, the protein bands which revealed the HSA cofactor and BSA were not observed. Instead, a white band appeared in the test wells below the degraded HA.

In this study oxidative DNA damage, measured by the levels of oxidised guanine nucleotide (8-oxoguanine), was elevated in samples which showed higher CML levels. Oxidative damage is caused when ROS become extremely elevated and/or when the antioxidant capacity of cells is unable to protect against ROS. AGE-related ROS generation can happen in a number of ways. Firstly, AGE intermediates can cause ROS generation during the glycation process as well as independently of AGE formation (Amicarelli *et al.,* 2003). Therefore the process of CML formation seen here is likely to be triggering ROS formation, such as has been demonstrated in endothelial cells, and which is inhibited in the presence of radical-scavenging antioxidants (Liu *et al.,* 2013). Secondly, another major source of ROS is from AGEs binding to their receptor, RAGE, on the cell membrane. This

activates NFκB-induced expression of pro-inflammatory genes and also of RAGE, leading to a sustained inflammatory response. AGE-RAGE binding is important in AGE pathology in various tissues but RAGE has also been located on the sperm membrane and its expression positively correlates with DNA damage (Mallidis *et al.,* 2007; Karimi *et al.,* 2012). Importantly, both of these parameters are elevated in the sperm of diabetic men (Karimi *et al.,* 2012). Although mature sperm are transcriptionally quiescent, which may suggest a limited capacity for NFκB cell signalling, immature sperm still have active gene expression and would be vulnerable to ROS generation through this pathway. This is in accordance with the results from Karimi *et al.* (2012) who found that RAGE expression was more prominent in the immature cells of the seminiferous epithelium. Furthermore, although almost no human studies have investigated NFkB in sperm, a mouse study has shown that NFkB is activated in stage-specific manner in spermatogenesis.

Despite there being changes in the level of oxidative DNA damage, ROS levels were not different between sperm incubated with different compounds. This may be due to the fact that sperm had been incubated for an extensive period of time, in which case ROS generation would be at a maximum across all samples as cell death began to occur. Furthermore, as the sperm were separated from the seminal plasma, which is the source of antioxidants and radical scavenging enzymes important for fertility (Lewis *et al.,* 1997; Khosravi *et al.,* 2014), protection again ROS would be low, allowing levels to accumulate. Finally, the lack of difference in ROS levels might be explained by the fact that ROS are unstable, so the sensitivity of the assay may insufficient for determining differences between these cells.

Sperm are particularly susceptible to DNA damage as they lack the repair mechanisms usually present in cells. Furthermore, the glyoxalase pathway, which detoxifies AGE intermediates through the action of Glyoxalase 1 (Xue *et al.,* 2011), has not been identified in sperm. This makes sperm particularly vulnerable to DNA damage which occurs primarily due to oxidative stress in these cells (Aitken *et al.,* 2010). The implications of such damage for sperm *in vivo* are reduced cell function, ultimately leading to poorer fertilisation

capacity. In accordance with this, 8-oxoguanine levels have been linked to lower pregnancy rates independent of semen quality (Loft *et al.,* 2003; Mulholland *et al.,* 2011). This supports the results here which did not see a significant change in the motility of sperm treated with GO where oxidative damage was highest. Oxidative DNA damage can occur in normal sperm as well as in abnormal sperm, and as ART treatment does not involve the analysis of DNA damage, this may have consequences for embryonic development and offspring health (Zhang *et al.,* 2008; Erenpreiss *et al.,* 2008; Oleszczuk *et al.,* 2013).

5.3.1 Conclusion

AGEs have important roles in the pathologies of ageing and disease. This study found that AGEs are formed on human sperm when exposed to reactive AGE intermediates *in vitro* and leads to oxidative DNA damage in these cells. As high levels of AGE and RAGE are found in the seminiferous epithelium (Mallidis *et al.,* 2009; Karimi *et al.,* 2012), it is possible that sperm are exposed to AGE modification and AGE-RAGE signalling during spermatogenesis. This could present a risk to older men or diabetics where intermediates and AGEs are elevated. The consequences of this for sperm function may not manifest as a change to standard semen parameters as this study found no inhibition of sperm motility in response to glycation, however evidence from other studies suggests that glycation-mediated oxidative damage could impair fertility (Oleszczuk *et al.,* 2013). Finally, although *in vitro* glycation elicited no change to cellular or recombinant sperm hyaluronidase function, the high levels of AGEs found on the sperm cell suggest that other sperm proteins, lipids or nucleic acids are modified.

Chapter 6

Final discussion

Elucidating the effects of lifestyle and environmental factors on fertility has become a major research question in recent years in light of the changing lifestyle habits that have profoundly transformed developed and developing countries (Popkin *et al.,* 2006). Some of the consequences resulting from these changes include the obesity and diabetes epidemics, exposure to environmental toxicants and an ageing population. These changes have paralleled the publication of numerous reports describing a decrease in semen quality in various populations of men, and the role of lifestyle and environment in this therefore requires investigation (Carlsen *et al.,* 1992; Auger *et al.,* 1995; Rolland *et al.,* 2013; Romero-Otero *et al.,* 2015; Centola *et al.,* 2016). Furthermore, lifestyle-induced changes to sperm quality may occur beyond the parameters observed by standard semen analysis, resulting in deregulate DNA methylation and increased oxidative DNA damage.

This study, which investigated semen quality and DNA methylation in men attending a fertility clinic, found that the BMI was negatively associated with sperm concentration, while alcohol consumption and having an unhealthy diet were positively associated with sperm concentration and sperm motility, respectively. Furthermore, wearing boxer shorts was negatively correlated with % immotile sperm. Exposure to glues, adhesives and resins was correlated with an increase in global methylation while ex-smokers showed a higher level of *SNPRN* methylation than non-smokers. Global DNA methylation was negatively correlated with fertilisation rates in IVF patients while BMI was negatively correlated with fertilisation rates in ICSI patients. Overall, concentration and % progressive motility were associated with higher fertilisation rates in ICSI patients and % progressive motility was associated with higher cleavage rates in IVF patients. An investigation into the effects of glycation on sperm as a model for diabetes and obesity showed that CML is formed on sperm incubated with AGE intermediates in

as little as two hours and this was linked to an increase in oxidative DNA damage. However, sperm parameters and levels of sperm-CML were not different between obese and normal weight men in this study.

A number of studies have investigated the complex relationship between lifestyle, DNA methylation, sperm parameters and ART outcomes with varying results. This study showed that increased global DNA methylation was present in men exposed to glues, adhesives or resins, and this also positively correlated with % immotile sperm and negatively correlated with fertilisation rates in IVF patients. Many epoxy resins, as well as widespread everyday-use polymers, contain the endocrine disruptor Bisphenol A (BPA). High levels of urinary BPA have been associated with decreased semen parameters in exposed factory workers compared to non-exposed factory workers in China (Li *et al.,* 2011), and therefore the components of these glues, adhesives and resins could be a reason for the abnormal methylation seen in the men in this study. In terms of the effects of EDCs on sperm methylation, BPA exposure has previously been linked to a decrease in global methylation in sperm, measured using *LINE-1* methylation as a proxy (Miao *et al.,* 2011), however this is the opposite trend to that seen in this study. Indeed, our results contradict many existing studies which report a decrease in global methylation in response to environmental stress, i.e. exposures, which is often associated with an increase in DNA damage (Montjean *et al.,* 2015; Tunc and Tremellen, 2009). Of particular relevance, Meeker *et al.* (2010) found higher levels of DNA damage in the sperm of men exposed to BPA. The mechanism behind this is that the relaxing of chromatin in response to demethylation makes DNA vulnerable to oxidative attack, causing DNA damage. Furthermore, as one of the major roles of methylation is in the suppression of transposable elements to prevent their movement about the DNA strand, a decrease in global methylation could deregulate this and contribute further to genomic instability. Studies have shown that this genomic instability is associated with demethylation and can have negative implications for fertilisation rate, in contrast to that presented in this study, which found a decrease in fertilisation rates with increasing global methylation in IVF patients. Despite this logical mechanism for the effects of

global hypomethylation, other groups have shown that exposure to certain hazards, such as ionizing radiation, can increase global methylation, concurrent to the findings of this study (Kumar *et al.,* 2013).

The world is currently in the midst of an obesity epidemic, with around 13% of the world's population being obese (World Health Organization, 2015). Obesity gives rise to numerous comorbidities of which male infertility is thought to be one. In this study, few lifestyle factors were associated with sperm parameters and ART outcomes, however BMI was strongly negatively correlated with sperm concentration and fertilisation rates in ICSI patients. The link between obesity and low sperm quality has been reported elsewhere (Sermondade *et al.,* 2013), although MacDonald *et al.* (2013) found no such association. Mouse studies have been conclusive of findings in support of these associations (Bakos *et al.,* 2011). One of the consequences of obesity for sperm is thought to be the disruption of DNA methylation and therefore gene expression, leading to cellular dysfunction. This was illustrated in a recent human study which showed that men who underwent rapid weight loss following bariatric surgery showed completely remodelled sperm methylome (Donkin *et al.,* 2016). However, the study did not find significant differences in methylation of any of the imprinted genes with BMI, which is in accordance with the findings here, rather the effected genes were already known to be implicated in the severe onset of obesity. Alternatively to methylation, obesity could be having an effect on sperm by causing DNA damage. Our *in vitro* glycation assay presented a potential model for investigating obesity-related risks to sperm, namely exposure to reactive AGE intermediates and AGE compounds themselves, which are elevated under hyperglycaemic conditions, a metabolic characteristic of obesity. The major causes of DNA damage in sperm are oxidative stress and poor chromatin compaction (De luliis *et al.,* 2009) and the glycation model showed that CML formation was accompanied by an increase in oxidative DNA damage. This is in accordance with other studies that have found higher levels of DNA damage in the sperm of obese men (Dupont *et al.,* 2013) and with our sister study which used a similar population of men at the same fertility clinic and found a non-significant increase in DNA damage in

the obese group (Altakroni, 2015). In the present study, BMI was also found to be negatively correlated with fertilisation rates in ICSI patients, during which treatment sperm bypass the normal sperm selection processes, suggesting that oxidative DNA damage could be a mechanism behind this failed fertilisation. In support of this idea, DNA damage has been shown to be negatively associated with ART outcomes in a small number of human studies, and is thought to be more relevant following ICSI treatment (Simon *et al.,* 2011; Simon *et al.,* 2013; Jin *et al.,* 2015; Loft *et al.,* 2003; Zini *et al.,* 2011).

As AGEs have such widespread damaging effects on various tissue types (Goldin *et al.,* 2006), it is likely that they might also influence reproductive function. AGEs are primarily a function of age and hyperglycaemia, and therefore pose a significant risk to the health of the world's ageing population in which the prevalence of high-sugar diets is negatively affecting global health trends (Siervo *et al.,* 2014). Additionally, as delaying childbearing has become a common life choice for couples, the risks of AGE-accumulation on fertility may be greater in these cases. In the light of the claims of decreasing semen quality, AGEs are a possible contributing factor to these trends. Although numerous mouse models have been used for glycation, only one to date has investigated the effects on male reproduction (O'Neill *et al.,* 2010). However, this study simply supported existing human studies which show the localisation of CML on sperm and in the reproductive tract (Mallidis *et al.,* 2009; Karimi *et al.,* 2011), but did not investigate the effects on fertilisation capacity or pregnancy outcomes. In women undergoing ART, studies have shown that AGE accumulation is associated with reduced oocyte quality, lower fertilisation rates and poor embryonic development (Jinno *et al.,* 2011) and is thought to be one of the causes of ovarian ageing (Stensen *et al.,* 2013). The presence of AGEs on sperm, particularly DNA adducts, may therefore also have an effect on the embryo. Although this study did not identify on which biomolecules AGE-adducts were forming, as there was a high level of oxidative DNA damage, it is likely that there would also have been DNA adduct formation and intermediate-adducts which have been described *in vitro* (Frischmann *et al.,* 2005). What is more, as DNA adducts

have been found to be elevated in sperm of infertile men, this therefore warrants further investigation (Horak *et al.,* 2003; Gaspari *et al.,* 2003).

One of the major findings of this study was that prepared sperm and neat semen show distinct methylation patterns, with prepared sperm showing significantly higher methylation levels across all genes as well as for global methylation. Preparation of sperm for ART involves the removal of non-sperm cells, debris and immotile and immature sperm, and results in a purified population of high quality sperm. Considering the implications discussed with regards to the instability of the genome in a hypomethylated state, these findings fit with the idea that these prepared sperm which exhibit good general sperm characteristics (i.e. mature and motile) also contain quality DNA. This is in accordance with our sister study that found DNA fragmentation was lower, and therefore genomic integrity higher, in prepared sperm than neat semen (Altakroni, 2015). However, there is a disparity here against our other finding that global methylation was positively correlated with % immotile sperm as these cells are removed during the preparation process. There were not sufficient prepared sperm samples with DNA methylation analysis to determine whether methylation of these cells used for ART were associated with the outcomes. Laurentino *et al.* (2014) found that sperm exhibit methylation mosaicism where discrete populations of sperm from infertile men show abnormal methylation, in comparison to fertile men who show a generally homogeneous methylation pattern in cells of a single sample. Therefore, density gradient centrifugation of samples is likely to result in the isolation of sperm with a distinct methylation profile from the neat sample, which limits the validity of using neat semen analysis when considering ART outcomes, although these analyses are still insightful.

One of the main aims of this study was to determine the effects of lifestyle and environmental factors on imprinted gene methylation, as these factors are the target of much of the research on the sperm methylome. The reason for such attention is due to the crucial role of these genes in embryonic parent-of-origin gene expression. As these genes avoid the first round of epigenetic reprogramming that occurs following fertilisation (Reik *et al.,* 2001), it is hypothesised that aberrant methylation marks harboured from the

parental gametes could affect embryonic development and consequently offspring health. The epigenome is responsive to environmental cues which confers the ability of cells to respond to their environment, and which also makes cells vulnerable to damage. In sperm, DNA methylation has been shown to be affected by age, obesity, smoking and other environmental toxicants (Jenkins et al., 2014; Ding et al., 2015; Xu *et al.,* 2013; Miao et al., 2014). In this study, imprinted genes that showed variation with lifestyle and environment were *SNRPN* and *H19*. These genes showed elevated methylation levels in men who were ex-smokers and men who were exposed to glues, adhesives and resins, respectively, despite not being significant following multivariate analysis, and this has not been shown elsewhere. Another reason for the particular interest in imprinted genes is that aberrations are associated with poor sperm quality. Several studies have described aberrant imprinted gene methylation in the sperm of infertile men (Hammoud *et al.,* 2010; Houshdaran *et al.,* 2007; Marques *et al.,* 2008; Poplinski *et al.,* 2009), however imprinted gene changes do not always manifest in changes to semen parameters. In accordance with this, our study found that only *MEG3* was negatively correlated with sperm progressive motility, while *H19, SNRPN* and *PLAGL1* were not associated with semen parameters. The fact that changes in DNA methylation may not always be reflected in a change in semen parameters is important as sperm DNA quality is not assessed before ART. There is a growing amount of evidence arguing for routine DNA damage testing, which can be carried out using various assays such as Terminal Deoxynucleotidyl Transferase-mediated Nick End Labeling (TUNEL) (Sun *et al.,* 1997), Single Cell Gel Electrophoresis (COMET) assay (Ribas-Maynou *et al.,* 2012) or the Sperm Chromatin Structure Assay (SCSA) (Bungum *et al.,* 2011), however markers that definitively indicate adverse sperm DNA methylation have not yet been identified, and therefore do not warrant routine clinical use.

One candidate gene for the determination of sperm quality is *DAZL.* The present study found that *DAZL* methylation was consistently highly negatively correlated with sperm concentration and was a characteristic of all oligozoospermic men, which is supported in other studies (Navarro-Costa *et*

al., 2010; Li *et al.,* 2013). *DAZL* expression is controlled by methylation at its promoter and is essential for spermatogenesis and spermiogenesis; if the causes behind deregulated *DAZL* methylation and gene silencing were identified, this could be an important therapeutic target. The lifestyle and environmental exposures investigated here did not show any associations with differential *DAZL* methylation, although exposure to metal dust and fumes was almost significantly associated with an increase in methylation. No human studies have investigated the effects of environmental exposures on *DAZL* expression, however, one mouse study found that low dose exposure to an endocrine-disrupting pesticide, methoxychlor, downregulated expression of *DAZL* among other spermatogenic genes resulting in disruption of sperm differentiation (Du *et al.,* 2014). Given the importance of *DAZL* expression during germ cell development, in which it inhibits pluripotency in PGCs and prevents differentiation to somatic cell lineages while inducing apoptosis if this does occur (Chen *et al.,* 2014), environmental modifications during the PGC development period would negatively affect adult male offsprings' fertility. In this study *DAZL* methylation was not related to IVF or ICSI outcomes. Therefore, although this gene has critical roles for spermatogenesis, it may have less significance for the developing embryo. In accordance with this, maternal-derived *DAZL* has been shown to be important during embryogenesis at the 2-cell stage while the contribution of paternal *DAZL* has not yet been determined (Cauffman et al., 2005). Overall, this work suggests that defining which lifestyle and environmental factors affect *DAZL* could provide an important target for interventions to improve male fertility, but to our knowledge, the use of sperm with aberrant *DAZL* methylation in IVF and ICSI treatment is not a risk for embryonic development.

Overall, DNA methylation tended to show an increase when men were exposed to factors considered to be damaging and in men with abnormal sperm, and this was in both imprinted and non-imprinted genes. For example, *DAZL* methylation was elevated in men with low sperm concentration and *MEG3* methylation was higher in men with lower % progressive motility. In combination with the global methylation results which

were elevated in response to environmental exposures and in men with high % immotile sperm, this implies that generally an increase in methylation is associated with negative outcomes. It is logical to suspect that the source of this hypermethylation is aberrant *de novo* methylation occurring during sperm development at various life stages, potentially caused by improper DNMT activation. DNMTs are activated during PGC development to establish new gender-specific imprints following the global demethylation that occurs in PGCs (Kaneda *et al.,* 2004), but their overexpression causes gene silencing and has been implicated in a number of cancers and in aberrant imprinting (Robertson *et al.,* 1999; Biniszkiewicz *et al.,* 2002). In fact, a recent study did investigate the correlation between DNMT expression and global methylation, and not only did they find a similar trend to that of this study in that global methylation was elevated in the sperm of men with impaired spermatogenesis, but this also correlated with an increase in *DNMT1* and *DNMT3a* expression (Jaiswal *et al.,* 2013).

Another candidate gene for deregulated methylation is *MTHFR.* The transcript of this gene, *mthfr*, is essential for the generation of methyl donors for methylation by DNMTs and therefore its disruption could affect the methylation of other genes. Indeed, aberrant *MTHFR* methylation has been implicated in infertility (Botezatu *et al.,* 2014) and hypermethylation of its promoter has been linked to hypomethylation of *H19* previously (Rotondo *et al.,* 2013), which therefore warrants further investigation. An alternative explanation for the higher levels of methylation described here is that there has been improper erasure of methylation during the second wave of developmental reprogramming in PGCs (Mayer *et al.,* 2000), as opposed to *de novo* methylation, in which case these aberrant methylation marks would have occurred *in utero* during PGC development. This would indicate that in effect it is maternal lifestyle factors and environmental exposures that are the cause of aberrant sperm methylation in adult men, rather than these being acquired paternal epimutations. Animal models have been crucial for exploring these scenarios, and *in utero* exposure experiments have illustrated the importance of the vulnerable time of PGC formation for adult sperm quality (Sobinoff *et al.,* 2014). This has been supported in a small

number of human studies in which subfertility has been linked to parental exposures (Axelsson *et al.,* 2013; Cirillo *et al.,* 2011).

These data lead on to the question of whether these epigenetic abnormalities have the potential to be inherited transgenerationally, and forming the crux of these investigations. Longitudinal health studies have suggested that the paternal germline can harbour both beneficial and harmful marks that are influence the health of future generations (Bygren *et al.,* 2001; Northstone *et al.,* 2014). This is supported by mouse studies which highlight an important role for male exposures on offspring health, for example in the nutritional availability of fathers on the metabolism of offspring (Anderson *et al.,* 2006; Binder *et al.,* 2012; Fullston *et al.,* 2013; Jiminez-Chillaron *et al.,* 2009), and in relation to other factors such as stress (Mychasiuk *et al.,* 2013) and toxins exposure (Mao *et al.,* 2015). Some studies have found that aberrant methylation is involved in this (Soubry *et al.,* 2015; Kobayashi *et al.,* 2009). In this study, the first indication of whether epimutations could be heritable was in looking at the effects of ART outcomes. Although our study showed that global DNA methylation was elevated in the sperm of men with lower fertilisation rates, we did not see an association with pregnancy outcomes which would suggest that sufficiently accurate epigenetic reprogramming had occurred during development, and which would have erased aberrant paternal methylation patterns. This robust reprogramming has been illustrated in animal studies in which epigenetic aberrations caused by parental exposures are prevented in the embryo and offspring (Iqbal *et al.,* 2015; Hesson *et al.,* 2015).

6.1 Conclusion

In conclusion, while the data presented in this study does not show a causative relationship between lifestyle and environment and changes to sperm parameters, DNA methylation or ART outcomes, some of the variation within the study population may be explained by this. It seems that small changes to lifestyle and environment may not drastically improve semen

parameters or the success rates of those using ART, however, improvements to DNA methylation and reduction in oxidative DNA damage may be achievable. This is critical in the advent of increasing use of ART treatment, where DNA integrity is not currently considered for analysis. As some studies have reported an increase in imprinting disorders in children born through ART (Lazaraviciute *et al.,* 2014), and as DNA modifications may be heritable through the paternal germ line (Laubenthal *et al.,* 2012), it is important that possible improvements via lifestyle and environment are made known.

6.2 Future work

1. To further investigate *MTHFR* and *DNMT* methylation and transcript levels in human sperm. These are functionally important genes for the methylation process, and this may shed light on the mechanisms behind changes in DNA methylation and particularly the overall increase in methylation seen here.

2. To investigate the correlations between DNA methylation and DNA damage to determine whether these processes are linked, leading to a collective effect on genomic integrity. To determine whether the combination of both methylation defects and DNA damage could confer a more severe infertile phenotype and poorer ART outcomes.

3. Use an aged, obese or diabetic mouse model to determine the effects of glycation and AGE accumulation on sperm function and on fertilisation capacity of mouse oocytes and embryonic development. In humans, to determine whether sperm AGEs are correlated with serum AGEs and to define the protein, lipid and DNA adducts forming on sperm other than CML.

6.3 Study limitations

This study used bisulphite pyrosequencing to analyse CpG site methylation which is considered to be a robust and precise method for measuring DNA methylation. However, the fragment length for sequencing was limited to 50 bp, since we found that longer sequences with dense CpG sites or sequences with a high number of cytosines and thymines resulted in poorer reads. This limit on the number of CpG sites that could be analysed meant that potentially relevant sites could have been missed. The CpG site-specific analysis carried out in this study showed that most of the CpG sites within each region had the same kind of variation, i.e. positively correlated with the mean methylation across the region, except for *MEG3* which had one differential site and *NR3C1* in which the 5th CpG site was less strongly correlated with the mean. However, some studies do show that certain CpG sites are more relevant than others.

Participants were recruited for this study from a fertility clinic. A major limitation of this is that semen quality is likely to be lower than that of the general population, even considering that many couples will present to the clinic with female-factor infertility, therefore significant lifestyle and environmental effects will only be specific for this subpopulation. This is the main issue with all studies involving subfertile men. Furthermore, within men undergoing ART, there may be a difference between men who chose to participate in the research study and those who did not. Our sister study which analysed the same cohort of men that were included in our methylation analysis (chapter 3), but not for the questionnaire (chapter 4), found that sperm concentration and % progressive motility were higher in the research group compared to the group which underwent ART treatment but did not participate. Fertilisation rate was also higher in the research group, while pregnancy rate was higher in the non-participant group. Finally, in terms of men who decided to complete the questionnaire, semen parameters and clinical outcomes were not significantly different from men who did not complete the questionnaire (Altakroni, 2015).

The questionnaire format used in this study is one used widely for collecting public health data. However, self-reported data can often be under or over-stated. Upon consultation at the hospital, men were advised to improve their diet and to stop smoking. Therefore, many participants may not declare certain information on the questionnaire for fear of repercussions. Furthermore, those who participated in the questionnaire may have been aware of their semen quality. Additionally, the questionnaire did not include any information on the female partner's lifestyle habits, so the ART results were compromised to an extent, since maternal factors could not be controlled for. These may have included BMI, previous smoking, lifestyle exposures and oocyte quality.

There are some limitations with regard to the *in vitro* glycation study. These include that exposure of sperm to AGE intermediates may not be physiologically relevant as these compounds have not been identified in the male reproductive tract. Furthermore, the concentrations used were higher than normal physiological levels. However, all *in vitro* studies must consider these limitations in that they cannot truly represent *in vivo* scenario. Finally, all *in vitro* studies on mature sperm have the same drawback which is that these cells are physiologically distinct from pre-ejaculate sperm and have a limited lifespan post-ejaculate. However, as a model to determine whether these cells may be vulnerable to damage by certain compounds, this is a valid investigatory starting point that future studies may take forward.

Appendices

Appendix 1: Lifestyle questionnaire

Study Number | |

Determinants of semen DNA damage in humans (SDD)

This study has been set up to investigate what factors may affect male fertility. Please work through the questionnaire filling in each question that applies to you, by ticking (√) the box or by writing in the space provided.

What is your date of birth?_____month _____ year

Date questionnaire filled in: _____day _____month _____ year

Please complete the details above, then turn over and answer the following questionnaire which consists of two parts. Both of them are about your job, lifestyle and diet. The first part is regarding the last 24 hours. The second part is about your life during the last three months.

Please read the questions carefully and give attention to the **bold** words. Answering this questionnaire should take about 15-20 minutes. Most of the questions have YES or NO answers, or questions where you need to tick a box √ or write a number or a word. There is one question about your job where we will ask you to answer in detail.

Study Number | |

Please answer the following questions **about the last 24 hours:**

1. Smoking:

1.1. Have you smoked in ***the last 24 hours***? Yes ☐ No ☐

1.2 If yes, what did you smoke (cigarettes, pipe,……..)?

1.3 How much of the above did you smoke (*e.g.* 5 cigarettes)?

If No,

1.4. In ***the last 24 hours,*** were you exposed to other people's cigarette smoke? Yes ☐ No ☐

1.5. If yes, how long were you exposed for? ☐ Hours

2. (***Before today***) when did you last ejaculate?

3. Attendance at Work

3.1. What is your current job title (e.g. Decorator)?

3.2. Have you been at work during the last 24 hours Yes ☐ No ☐

3.3. If 'yes', was this typical of your normal work Yes ☐ No ☐

If No, please give more details:

4. Are you a vegan or a vegetarian? Yes ☐ No ☐

5. In the last 24 hours did you have any of these food**:**

5.1. Chicken or other poultry Yes ☐ No ☐

If yes, please state how cooked (fried, smoked, barbeque, grilled, other …)

5.2. Red meat (beef, lamb, ham, sausages ……) Yes ☐ No ☐

If yes, please state how cooked (fried, smoked, barbeque, grilled, other …)

5.3. Fish Yes ☐ No ☐

If yes please state how cooked (dried, frozen, fried, smoked, other ….)

Determinants of semen DNA damage in humans (SDD)

Study Number

Please answer the following questions (Q1→ Q20) about your job, health, lifestyle and diet **during the last three months**

Your job during the last three months:

Please write in the table in the next page the type of work you have done in your job during the ***last three months***, please, if you have been in two or more jobs during the last three months, mention all of them. To help you, we have given some examples of what other men have written in a previous study. Please answer this question even if you were not exposed to any hazard in you job (for example office job).

Examples	**Job Title**	**Type of company** (what did they do)	***During the last three months,*** **What were the main tasks you did in your job?** ***And what were the hazards (chemicals, radiation,....)*** *you were exposed to (if applicable)?*	**Type of job (full or part time)**	**Did you have to wear any sort of personal protection during your job**
1	Telephone account manager	Airline	Meetings with clients over telephone, lots of sitting down.	Full-time	None
2	Sales assistant	Garden centre (Gardening)	1- Selling pesticides, weed killers, fertilisers. 2- Making up pesticides, weed killers, fertilisers for use in garden centre. 3- Cleaning up spillage.	Part-time	Protective clothing, respirator, gloves, coverall
3	Pipefitter	Plumbing firm	1- Installing industrial pipe work, gas, water, steam, compressed air and this includes soldering. Currently I use flux which is lead free solder.	Full-time	None
4	Electrical engineer	Nuclear power planet	Executing, evaluating, delivering and managing complex engineering and / or cross functional tasks or programmes of work, recognising stakeholder and customer requirements.	Full-time	Protective clothing, gloves……
5	Researcher	University	Working in the lab and dealing with chemicals and biological materials.	Full-time	Protective clothing, gloves
6	Decorating	Domestic decorating	1- Wallpaper decorating: I used adhesives, fillers, putty. 2- Painting: oil paint, white spirit. 3- Decorating involves lacquers and silicones.	part-time	Gloves

2

1. Please complete the table below and write as much as details to describe ***your jobs in the last three months***.

If you had more than one job ***during the last three month*****, please mention all of them**	**Job Title**	**Type of company** (what did they do)	***During the last three months,*** **What were the main tasks you did in your job?** ***And what were the hazards (chemicals, radiation,....)*** *you were exposed to (if applicable)?*	**Type of job (full or part time)**	**Did you have to wear any sort of personal protection during your job**

3

2. ***During the last 3 month, at your job and/or at home***, have you worked with, or been exposed to any of the chemicals or physical agents in the table below.

Chemicals or Physical agents at *job/home*	**Please put √ in shaded boxes for Yes or NO**		
	Yes √		**No** √
Metal Dust or Fumes			
Pesticides			
Weed killers (herbicide)			
Fertilisers			
Oils or Greases			
Detergents or Soaps (at job only)			
Glues, Adhesives or Resins (for example, decoration)			
Paints, Varnishes or Lacquers			
Printing Inks or Dyestuffs			
Dry Cleaning Fluids			
PVC or plasticisers (at job only)			
Other Solvents (at job only) **(For cleaning, degreasing, thinning, lubricating, mixing, etc.)**			
Extremely Hot Environments (at job, Sauna, bathing in hot water for long time)			
Heavily Vibrating Machinery, Equipment or Vehicles			
Non-Ionizing Radiation **(microwaves, lasers, or high voltage electrical power)**			

Your health:

3. ***In the last 3 months*** have you had any fever or flu? Yes ☐ No ☐

3.1. **If yes**, how long did it last? ☐ days

4. ***In the last three months*** Have you had any illnesses that have been caused or been made worse by your work? ☐ Number of illnesses

4.1. **If yes,** How would you describe this illness?	Please tick
1. Breathing or lung problems.	☐
2. Skin problems.	☐
3. Hearing problems.	☐
4. Stress, depression or anxiety.	☐
5. Headache and/or eyestrain.	☐
6. Heart disease / attack, other circulatory system.	☐
7. Infectious disease (virus, bacteria).	☐
8. Bone, joint or muscle problems which mainly affect:	
8.1. Arms, hands, neck or shoulder.	☐
8.2. Hips, legs or feet.	☐
8.3. Back.	☐

9. Other, please specify: ☐

5. Do you have a varicocele (Varicose veins in the scrotum) now? Yes ☐ No ☐

5.1. **If No**, Have you ever had a varicocele? Yes ☐ No ☐

Your lifestyle:

Smoking:

6. Are you — please tick one

A ***current*** smoker (*at least* ***one cigarette per day***) ☐

An ***ex-smoker*** ☐

A ***non-smoker*** (***never*** smoked) ☐

If you are a current smoker, please answer questions 6.1 → 6.3 then go to question 6
If you are an ex-smoker, please answer questions 6.3 → 6.7 then go to question 6
If you are a non-smoker, please answer questions 6.6 → 6.7 then go to question 6

6.1. What do you smoke? (please tick the boxes for all that apply)

Cigarettes ☐ hand rolled cigarettes ☐ cigars ☐ pipes ☐

Other ☐ please specify: ……

6.2. How much do you smoke ***a day on average*** (cigs/day, oz/day, other…)

_____

6.3. At what age did you **start** smoking regularly _____

6.4. At what age did you **stop** smoking? _____

6.5. What did you smoke? (please tick the boxes for all that apply)

Cigarettes ☐ hand rolled cigarettes ☐ cigars ☐ pipes ☐

Other ☐ please specify: ……

6.6. Are you exposed to cigarette smoke of other smokers at home, work, club…? Yes ☐ No ☐

If yes:

6.7. ***During the last three months,*** How many hours a week (on average) have you been exposed to the cigarette smoke of other people? ☐ hours

Drinking Alcohol:

7. Do you drink alcohol regularly? Yes ☐ No ☐
(***at least one drink per week***)

If yes:
7.1. ***During the last three months***, how much alcohol did you consume on average ***every week*** (please write a number in the boxes when applicable)?

☐ Pints of Lager ☐ Pints of beer ☐ Measures of spirits

☐ Glasses of wine Other, please specify ☐

Clothing:

8. Do you usually wear boxer shorts as underwear? Yes ☐ No ☐

Exercise:

9. *In the last seven days*, how many times did you do the following kinds of exercise for ***more than 15 minutes*?**

1. **Strenuous** exercise
(*exercise which makes your heart beat rapidly*) ☐ times

2. **Moderate** exercise
(*exercise which is not exhausting*) ☐ times

3. **Mild** exercise
(*exercise which required a minimum of effort*) ☐ times

10. *In the last seven days, during your leisure-time*,
how many times did you do strenuous activity for long ***enough to work up a sweat*** (so that your heart beats rapidly)? ☐ times

Your diet:

11. How would you describe your diet ***during the last three months***?

☐ Vegan ☐ Meat eater

☐ Vegetarian ☐ Meat and fish eater

☐ Fish eater

12. Please answer the following questions (**Table in the next page**) about what you usually eat and drink. Although the list looks a little bit long, it should not take more than 10-15 minutes to complete. Please read the instructions below.

- Please tick (*Never*) column if you have not eaten the food during the last three months.
- If you tick the wrong box by mistake or you want to change your answer, please put a ✕ on the wrong answer and a √ in the right box.
- In the last column *("How much do you normally eat **per serving**?")*, you can answer by using the number of unit or number of pieces. For example, 3 apples, 5 tablespoons, 3 sausages, 2 cup of tea, 1 bottle (500ml) of orange juice, 1 pint of milk, 5 slices of bread or toast, large, medium or small serving of broccoli, chicken, beef, ……. .
- **Examples:**

FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**?
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Chicken or other poultry ***(white meat)***					√			large, medium or small serving
Beef including mince				√				250g or large serving
Spinach	✕	√						Medium serving
Black tea						√		1 cup
Orange			√					250 ml or medium bottle of orange juice
Bok choy (Pak choi)	√							Medium serving

FOODS	How many times did you eat these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**?
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Chicken or other poultry ***(white meat)***								
Smoked chicken or other poultry								
Beef including mince								
Beefburgers								
Lamb								
Ham								
Bacon								
Sausage or chorizo								
Hot dogs or frankenfurter								
Liver								
Smoked red meat (red meat includes beef, lamb, ham, bacon,…)								
Very fried meat, barbeque, grilled meat (***red and white meat***)								
Fish (dried, frozen)								
Oily fish (fresh or canned e.g. tuna, salmon, sardines,…)								
Fried fish								

FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**?
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Smoked fish								
Oysters								
Dried shrimps								
Broiled or grilled squid								
Sauerkraut								
French fries, fried potatoes, chips								
Medium and very spicy food								
Food that has been wrapped in cling film (plastic film or wrap)								
Canned food (tuna, peas, beans, ……)								
Vegetable oil								
Soy flour, Soybeans, boiled, Tempeh, Soy meat.								
Full fat milk								
Semi-Skimmed milk								
Skimmed milk								
Skimmed/fat free milk								
Dried Milk								

FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**?
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Yoghurt (Low fat)								
Yoghurt (Natural)								
Eggs								
Cottage cheese								
Cheese (all types)								
Butter								
Orange (fruit, juice)								
Blackberry (fruit, juice)								
Blueberry (fruit, juice)								
Cherry (fruit, juice)								
Kiwi (fruit, juice)								
Black grape								
Aubergine								
Onion, Leek								
Beans								
Asparagus								
Beet red (red beet or beetroot)								

FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**?
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Chinese Cabbage								
Cabbage (other types)								
Broccoli								
Spinach								
Lettuce								
Parsley								
Bok choy (Pak choi)								
chick peas, dried								
Almonds								
Peanut								
Breakfast cereal								
Biscuits or Cookies								
Toast								
Bread (all types)								
Pies, cakes and puddings								
Wheat germ (whole wheat bread)								

FOODS	How many times did you eat or drink these foods in ***THE LAST THREE MONTHS***?							How much do you normally eat **per serving**?
	Never	Less than once a month	1 – 2 times per month	Once a week	2 – 3 times per week	4 – 6 times per week	Every day	
Wheat bran								
Rice								
Ice cream								
Chocolate								
Black tea (cups)								
Green tea (cups)								
Coffee (cups, Not decaffeinated)								
Coca-Cola (or non brand coke) Not decaffeinated								
Pepsi (or non brand) Not decaffeinated								
Red Bull (or non brand) Not decaffeinated								
Vitamin B_6 tablets								
Vitamin E								
Zinc tablets								
Selenium tablets								
L-arginine tablets								
L-Carnitine tablets								

13. Where do you get most of your drinking water at home?

☐ Mains tap water ☐ Bottled water ☐ Other eg well or spring

14. How is your milk usually packaged?

☐ Plastic ☐ Glass

☐ Carton ☐ Other, please state ☐

15. How is your vegetable oil usually packaged?

☐ Plastic ☐ Glass ☐ Other, please state ☐

16. How are your beverages (juices, fizzy soft drink) packaged?

☐ Mostly glass ☐ Mostly plastic ☐ Mostly cartons

☐ No packaging ☐ Can ☐ Other, Please state ☐

General questions:

17. What is your level of education? (Please tick one)

☐ Primary school ☐ Secondary school ☐ GCSE or equivalent

☐ A Level ☐ Undergraduate university ☐ Postgraduate university

18. What is your **height** and your **weight** now?

Height ☐ cm or ☐ feet or ☐ inches

Weight ☐ Kg or ☐ stone

Ethnicity

19. Which ethnic group do you consider you belong to?

☐ White	☐ Indian
☐ Black-Caribbean	☐ Pakistani
☐ Black-African	☐ Bangladesh
☐ Black-Other, Please describe _____	☐ Chinese
☐ Other Ethnic Group, Please describe _____	

If there is any information about your job, lifestyle, diet or habit that you think may be of relevance to this study, please use the space below.

_____

_____

_____

Many thanks for filling in the questionnaires. Please put the completed questionnaire in the box on the nurse station on the ward.

Appendix 2: Semen DNA Damage Consent Form

Central Manchester University Hospitals NHS
NHS Foundation Trust

Questionnaire consent form (MALES ONLY)

Study Number **SDD**:

Sperm DNA Damage (SDD)

Principal investigator: Professor Daniel Brison

Thank you for reading the patient information sheet about our research
(Origins and biological significance of DNA methylation and DNA damage in human sperm).

If you would like to take part, please initial all boxes:

1. I have read and understood the information sheet and have been given a copy to keep. I have had the opportunity to ask questions about the research.	
2. I agree to answer a questionnaire about my recent jobs, lifestyle and diet. I understand that participation is voluntary and I am free to withdraw my consent at any time without giving a reason and that if I withdraw this will not affect my medical treatment or legal rights.	
3. I understand that any information obtained in the questionnaire will be confidential and that my information will be kept anonymous and identified by a study number only.	
4. I agree that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the University of Manchester, from regulatory authorities or from Central Manchester University Hospitals NHS Foundation Trust, where it is relevant to my taking part in this research.	
5. I agree that relevant sections of my medical notes and data collected during the study may be looked at by research team for research purposes only.	
6. I have been given contact details for the research team and have been told how to access further information if required.	

…… …… ……
Name of Male (Print) Date Signature

…… …… ……
Accepted by Date Signature

Version 1.0 16/04/2014

Appendix 3: Clinical Research Network Support Letter

NHS
National Institute for Health Research
Clinical Research Network

Dear Professor Brison

Re: Sperm DNA Damage

IRAS Ref: 155745 Study ID: 16938

We are pleased to inform you that the above study has been assessed as eligible for consideration for CRN support. This study has been included on the National Institute for Health Research Clinical Research Network (NIHR CRN) Portfolio. Your unique Portfolio Study ID number is detailed above and can be used to search for the record for this study on the NIHR CRN Portfolio.

The NIHR is committed to providing the CRN support requirements needed for your study to be successfully delivered in the NHS, this includes access to a local network of dedicated, skilled research support staff including research nurses and other allied health professionals, who can help identify eligible patients, arrange consent to participate in the study and monitor patients as they progress through the study. Other ways of ensuring the success of the study in the NHS include access to pharmacy, imaging and pathology services and the possibility of securing protected time for NHS staff to conduct research.

The Clinical Research Network comprises of 15 Local Clinical Research Networks that cover the length and breadth of England. Each Local Clinical Research Network delivers research across 30 clinical Specialties. Your study will be supported by the Clinical Research Network and its associated Specialties, which are here to support you throughout the life of your study and can provide you with help and advice if you encounter any problems which adversely affect the start-up and subsequent recruitment into the study.

Your study has been allocated to the NIHR Clinical Research Network**: Greater Manchester**
Information on how to access CRN support can be found on the website:
http://www.crn.nihr.ac.uk/greater-manchester/

Your study has been allocated to the **NIHR Clinical Research Network: Reproductive Health and Childbirth.** Information on this Specialty can be found on the website at
http://www.crn.nihr.ac.uk/reproductivehealth/
and an up-to-date contacts list here:
http://www.crn.nihr.ac.uk/reproductivehealth/contacts/

Recruitment Data

If your study is accessing CRN support, you are required to upload recruitment data on a monthly basis. This is essential to ensuring that the NIHR can report accurately to the Department of Health the number of people actively participating in research. Recruitment data is measured against key performance indicators which are used to monitor the success of the Clinical Research Network and will feed into the process of allocating future funding for NHS infrastructure for research to Clinical Research Networks. This ensures that infrastructure resources are directed to where they are required for the most patient benefit. The reporting of recruitment data also helps the Specialty to identify

Delivering research to make patients, and the NHS, better

studies which are struggling to recruit and to provide support for these studies. If you are required to upload recruitment data you will be sent instructions on how to do this.

International Standard Randomised Controlled Trial Number (ISRCTN)

One of the Department of Health's policies is to encourage transparency and promote public access to information about research and research findings affecting health and social care. Accordingly, the Department of Health strongly encourages voluntary registration of both interventional and observational clinical research studies on its preferred public register, the International Standard Randomised Controlled Trial Number (ISRCTN) Register, which is the World Health Organization's primary registry for the UK and is administered by Current Controlled Trials Ltd.

The NIHR Clinical Research Network has developed a process which enables automatic and seamless registration of all new UK Clinical Research Network (UK CRN) Portfolio studies via the UK CRN Portfolio.

New non-commercial studies with an interventional component included on the National Institute for Health Research (NIHR) Clinical Research Network (CRN) Portfolio, which are not currently registered with ISRCTN or ClincalTrials.gov, will be registered 'free' if they choose to register via the UK CRN Portfolio functionality.

Observational, industry-sponsored and devolved administrations studies (i.e. studies without English sites) are encouraged to use the UK CRN Portfolio functionality to register with the ISRCTN; however for these studies ISRCTN registration will incur a fee payable by the relevant organisation/company and invoiced directly from Current Controlled Trials Ltd.

To register for an ISRCTN via the UK CRN Portfolio functionality, log onto the Portfolio database via https://portal.ukcrn.org.uk/login/ and select 'yes I wish to register for an ISRCTN' and complete the extended minimum dataset required for ISRCTN registration.

The details of your study will be forwarded to Current Controlled Trials and the ISRCTN editorial team will contact you in due course. **Please do not apply directly to Current Controlled Trials if you are registering for an ISRCTN via the UK CRN Portfolio.**

Acknowledgement of Clinical Research Network support

Acknowledgement of Network support must be made when publishing study findings.

It is your responsibility to ensure that the following standard text is used to acknowledge the support of the Clinical Research Network when publishing your study findings in peer-review journals, or any other form of publication:

[Research team or organisation] acknowledge the support of the National Institute for Health Research Clinical Research Network.

Please do not hesitate to contact the CRN Portfolio team should you require further information. Email: portfolio.crnH@nihr.ac.uk

Best Wishes

Jonathan Gower

Dr Jonathan Gower
Research Delivery Director

National Institute for Health Research Clinical Research Network
16 Clarendon Place, Leeds, LS2 9JY

Tel: 0113 343 0401
Fax: 0113 343 1928
Email: jonathan.gower@nihr.ac.uk

Web: www.crn.nihr.ac.uk

Appendix 4: CMFT R&D Approval Letter

Central Manchester University Hospitals NHS
NHS Foundation Trust

Research Office
Rehab. Corridor, Junction 3
Manchester Royal Infirmary
Oxford Road
Manchester M13 9WL
Tel: 0161-276-3565
Fax: 0161-276-5766

Professor Daniel Brison
St Mary's Hospital
Central Manchester Foundation NHS Trust
Oxford Road
Manchester
M13 9WL

Dear Professor Brison

PIN: R03617
Cost Code:TBC
CSP Reference:155745
REC Reference: 14/NS/0082
Research Study: Origins and biological significance of DNA methylation and DNA damage in human sperm

Thank you for submitting the above study for NHS R&D permission. **Central Manchester Foundation Trust** is the Sponsor for this study which ***is*** on the NIHR portfolio.

I am pleased to confirm that the Research Office has now received all necessary documentation, and the appropriate governance checks have been undertaken. This letter is issued subject to the research team complying with the attached conditions, Trust SOPs, the DH Research Governance Framework, and any other applicable regulatory requirements. This approval is in relation to the documentation listed.

CMFT are required to report whether the research was initiated within 70 days or provide valid reasons for not doing so. The target date for this study is listed below;

- NIHR 70 Day from Valid Submission to 1st Patient Recruited: **12 August 2014**

Further information regarding the NIHR target can be found on the intranet.

Please update CRIMSON with the date when the first patient was recruited. If you or one of your team requires training on CRIMSON please contact Michael.Horrocks@cmft.nhs.uk

I would like to take this opportunity to wish you well with your research.

Yours sincerely

L A Broadfoot

Lorraine Broadfoot
Research Operations Manager

Date: 7 July 2014

cc. Caroline Leech Divisional Research Manager
Lucy Dwyer Senior Gynaecology Research Nurse

Documents Acknowledged/Approved

Document	Version	Date
NRES Approval Letter	Conditions Met	02 June 2014
IRAS Checklist XML [Checklist_02062014]		02 June 2014
Letters of invitation to participant	1.0	16 April 2014
Non-validated questionnaire -Semen DNA Damage (last 24 hours)	1.6	11 July 2012
Funding Award Letter		11 March 2014
Response to REC Provisional approval letter	1.0	29 May 2014
Participant consent form	1.0	16 April 2014
Participant information sheet (PIS)	1.0	16 April 2014
REC Application Form	155745/602609/1/70	30 April 2014
Research protocol or project proposal	1.0	16 April 2014
Summary CV for Chief Investigator (CI) – Daniel Brison		12 September 2013
Validated questionnaire [Semen DNA Damage (last 3 months)]	1.6	11 July 2012

Conditions of Approval:-

- All researchers involved in the study need to have received training appropriate to their role covering aspects of Research Governance or Good Clinical Practice (GCP). Trust policy states GCP training needs to be renewed every 3 years.
- The Research Office must be informed of: (please forward copies of amended documents by email)
  - The actual start date of the project
  - Any changes to the protocol throughout the course of the project
  - Any amendments sent to the MHRA or Research Ethics Committee
  - Any changes to the management of the project
  - Any extensions to the project, and associated additional funding, if applicable.
- The Research Office must be notified immediately of all Serious Adverse Events (SAEs) and Suspected Unexpected Serious Adverse Reactions (SUSARs) via email adverse.events@cmft.nhs.uk or Research Office fax: 276 5766 and/or by copy of official notification to the regulatory authorities (NRES, MHRA as applicable).
- All research taking place on CMFT Trust premises is subject to the Trust monitoring programme, either as part of the annual 10% audit requirement or "triggered" monitoring[1]. The Chief and/or Principal Investigator is required to make him/her self available for any monitoring visit, on a mutually agreed date.
- All Principal Investigators are required to complete and submit an annual self-assessment at the request of the Research Office.
- All Principal Investigators are required to provide recruitment (accrual) data to the Research Office monthly.
- The Research Office must be given a minimum three months' notice, in writing, if the Principal Investigator leaves the employment of CMFT Trust.
- The Research Office must receive immediate notification if the Principal Investigator is unable to continue to fulfil his/her duties as PI for other reason e.g. long-term sickness
- Any evidence of fraud &/or misconduct must be immediately brought to the attention of the Research Office either via the Incident Reporting system, or by direct communication.

Failure to comply with any of the above may result in withdrawal of approval for the project and the immediate cessation of the research. Persistent failure to comply may result in disciplinary action.

Appendix 5: Semen DNA Damage Protocol

Central Manchester University Hospitals NHS
NHS Foundation Trust

Determinants of semen DNA damage in humans (SDD)

Daniel Brison [1], Andrew Povey [2], Jill Stocks [2], Michael Carroll [3], Clare Nevin [3]
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1. Background:

Approximately 15% of all couples worldwide seek help from infertility clinics to enable them to conceive (1-3). Almost 25% of them are couples with unexplained infertility (1), while male factor infertility alone is believed to cause 25% of infertile couples (1, 3). During the last four decades, there have been conflicting reports about whether male fertility is in decline or not. A decline in fertile men of sperm concentration (4-8), sperm morphology (4, 8, 9), sperm motility (4, 8) or semen volume (6, 7) has been reported in some studies but not all (10-14). This inconsistency might due to selection bias, methodology, differences in measuring sperm quality between laboratories, environmental conditions (15) and regional differences (16). A decline in the ability to achieve pregnancy or an increase in the time to pregnancy are more important indicators than semen parameters but this decline in fecundity has not been confirmed yet. However, the number of couples undergoing assisted reproductive technology (ART) is increasing and this might indicate an increasing willingness to seek treatment or a possible decline in fertility in the general population.

If the latter, the decline might be due to changes in lifestyle, or occupational and environmental exposure to toxic agents, dietary factors, and personal behavior like smoking (17-19). However, the exact mechanisms by which these factors might affect male fertility are still not clear. One suggested mechanism is that these exposures may have a deleterious effect on sperm DNA integrity by inducing oxidative DNA damage, DNA alkylation, DNA single strand breaks (SSBs)

or double strand breaks (DSBs), and altering gene expression through chemical modifications of DNA through methylation and microRNA expression, or other types of damage which, in turn, might reduce male fertility by impairing spermatozoa function and its ability to fertilize an egg (20, 21). DNA damage in human sperm, even if it is not related to a decline in semen quality and fertility, is a critical issue since the sequences of this damage such as mutations or epigenetic modifications are potentially heritable and might well cause defects in the offspring.

2. Aims and objectives:

The overall objective of this project is to investigate the biological significance of DNA damage in relation to male fertility and reproductive outcome. Therefore, this research will:

- Study the correlation between the occupational and environmental exposure and sperm DNA damage including N7- methylguanine (N7-MedG), SSBs and DSBs, DNA methylation and microRNA expression
- Investigate the relationship between these types of damage and semen quality (sperm concentration, motility, morphology).

Compare DNA damage between the different types of infertile men and examine the contribution of the different types of DNA damage to the outcome of ART.

3. Methods:

3.1. Study design:

The study has a cross sectional design in which the population will be men of infertile couples who are attending the Department of Reproductive medicine at St. Mary's Hospital for ART.

3.2. Recruitment:

The process for recruitment is shown in (Figure 1).

When couples are referred by the GP to the hospital for the first time, they will be sent information about clinical treatment and ongoing research within the Department of Reproductive Medicine. On their first visit, a blood sample will be taken from the couple for routine testing. During their second visit to the hospital, couples will meet the clinical staff in the Department of Reproductive Medicine and be required to sign HFEA consent forms for treatment and storage of sperm, eggs and embryos and to state whether or not they are willing to be approached about research. Those who consent to being approached about research will be

referred to a research nurse working within the department (Figure 1, 2nd visit, step 1). The research nurse will then provide additional information (including a written patient information sheet) about the various ongoing research studies including a study entitled "In-vitro Development & Implantation of Normal Human Pre-embryos & Comparison with Uni & Poly-pronucleate Pre-embryos" (HFEA license R0026, V2 05/02/14). This study includes consent for the collection of semen samples to measure sperm DNA damage (Figure 1, 2nd visit, step 2). If the man consents to take part in this study, he will then be asked if he would also like to participate in a relevant sub-study which will involve the male partner completing a questionnaire about his job, lifestyle and diet (Figure 1, 2nd visit, step 3).

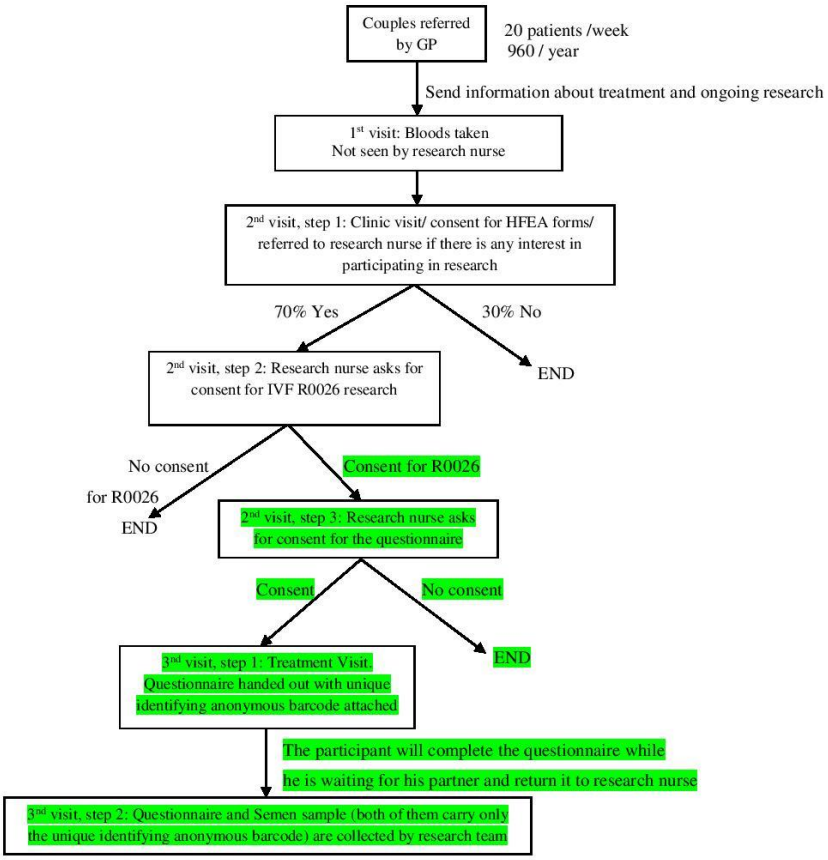

Figure 1 The process for participant recruitment. The steps (in green color) are related to the recent ethics application while other steps are already approved under ERP/91/078.

Those who choose to participate will be asked to sign a consent form to document this. One copy of the consent form will be given to the participant to retain for their personal records, one will be filed in their clinical notes, one will be photocopied and stapled within the couple's Integrated Care Pathway (ICP) booklet and the original kept in the study site file in accordance with Trust and GCP guidance. On the ICP copy, the letter 'Q' (for questionnaire) will be written. On the day prior to egg retrieval the embryologists will identify the patients in the study and write 'Q' on the Andrology request form and pass to Andrology. On the day of egg retrieval, a member of staff at the Andrology reception will identify participants who require a questionnaire and will hand the participants a copy of the questionnaire with a unique identifying anonymous barcode attached and ask him to complete it while he is waiting for his partner (Figure 1, 3rd visit, step 1). The semen sample will be labelled with the same unique identifying barcode. After completing the questionnaire, which will take around 15-20 minutes, the participant will return the completed questionnaire to a member of staff on the ward.

In the instance when a participant is not given a copy of the questionnaire to answer on the day of treatment in error, the questionnaire and approved covering letter (V1.0 06/03/13) will be posted out to the participant' home to ask him to answer the questionnaire and return it to the research team using the FREEPOST envelope provided.

3.3. Semen samples and questionnaire collection:

The participant will produce a semen sample (treatment sample) as a part of routine treatment cycle. Approximately, 100µl of the sample will be used to examine the semen parameters (semen volume, sperm concentration, motility, morphology, the presence of leukocytes) and also a sufficient part of the treatment sample (~1 ml) will be prepared for the treatment cycle (IVF, ICSI) and this is called prepared sperm.

After finishing the treatment cycle, the remaining treatment sample and prepared sperm will be collected by the research team and moved on ice in a sealed box to the lab (room T1.08 John Dalton Building, Chester Street, Manchester Metropolitan University . Before moving the

sample from the hospital to the lab, the participant name and any other information are removed from the sample container and the container is relabeled only with the date and the R0026 identifying number which is on the questionnaire the participant has completed. The research team will also collect the questionnaire related to the sample (Figure 1, 3nd visit, step 2) and obtain information regarding semen quality (sperm concentration, motility, morphology).

3.4. Analysis of sperm DNA damage:

DNA damage in the sperm will be investigated using the following techniques:

- Neutral Comet assay: to measure double strand breaks in both the treatment sample and prepared sperm (22).
- Immuno slot-blot: to measure the level of N7- methylguanine in sperm DNA in the treatment sample and prepared sperm (23).
- Bisulphite sequencing: to measure nucleotide-specific DNA methylation at imprinted genes.
- microRNA expression analysis by real time PCR: to identify epigenetic regulation of important developmental genes by microRNAs.

When the treatment sample and prepared sperm arrive in the lab, the treatment sample is centrifuged to separate the sperm from the seminal plasma. The seminal plasma is stored while the sperm are washed twice by PBS and then the sperm pellet is suspended in 1ml PBS.

Prepared sperm are also washed twice by PBS and the pellet is suspended in appropriate amount of PBS. Approximately, 5×10^5 sperm from each suspension are used for Comet assay while the rest are centrifuged and the sperm pellet is stored at -80˚C.

When ten sperm pellets have been collected, the DNA is extracted from each pellet (23) and then level of N7-methylguanine in the extracted DNA is determined by using the immuno slot-blot technique. Remaining DNA from each sample will be bisulphite treated and sequenced to measure DNA methylation (25).

From fresh sperm samples, RNA will also be extracted, reverse transcribed and real time PCR performed to analyse microRNA expression (26).

3.5. Statistics:

- The correlation between DNA damage results (double and single strand breaks, N7-methyguanine, DNA methylation, microRNA expression) and semen parameters (semen volume, sperm concentration, motility, morphology, the presence of leukocytes) will be investigated.
- The correlation between the (exposure) questionnaire data (job, lifestyle, diet) and DNA damage results and semen parameters will be examined.
- The association between DNA damage results and IVF cycle outcome (number of oocytes fertilized, embryo quality, abortion and successful pregnancy) will be investigated.

3.6. Power of the study:

We have calculated the number of patients required based on the N7-MeG levels observed in our previous study (24). In this study the mean (±SD) N7-MeG levels were 1.16±0.99 μmol/mol dG in patients. Assuming a power of 90% and an alpha of 0.05, to detect a 33% change in adduct levels (~0.38 μmol/mol dG) between, for example high and low red meat consumption would require approximately 140 subjects in each group i.e. a total of at least 280 patients. To detect a 50% change in adduct levels would require a total of 120 subjects (~60 in each group.) In general, 20 couples undergo ART in the Department of Reproductive Medicine every week (Figure 1). 70% of them consent for R0026 research which includes the use of the remaining semen sample after treatment. From these 70%, we might expect 50% will consent for the questionnaire study. Thus, from the 20 couples, seven are expected to participate in the study every week. To recruit 120-280 subjects would require 17-40 weeks.

To investigate DNA methylation and microRNA expression (alternative aspects of DNA damage), 120 additional samples will be required bringing the total sample size to 400. This allows for attrition at 40% which is considered realistic due to the study methodology, as a number of participants will not return completed questionnaires or have surplus sperm which can be used by the researchers. Both a completed questionnaire and sperm sample are required to assess the effect of environmental factors upon sperm DNA and answer the study question.

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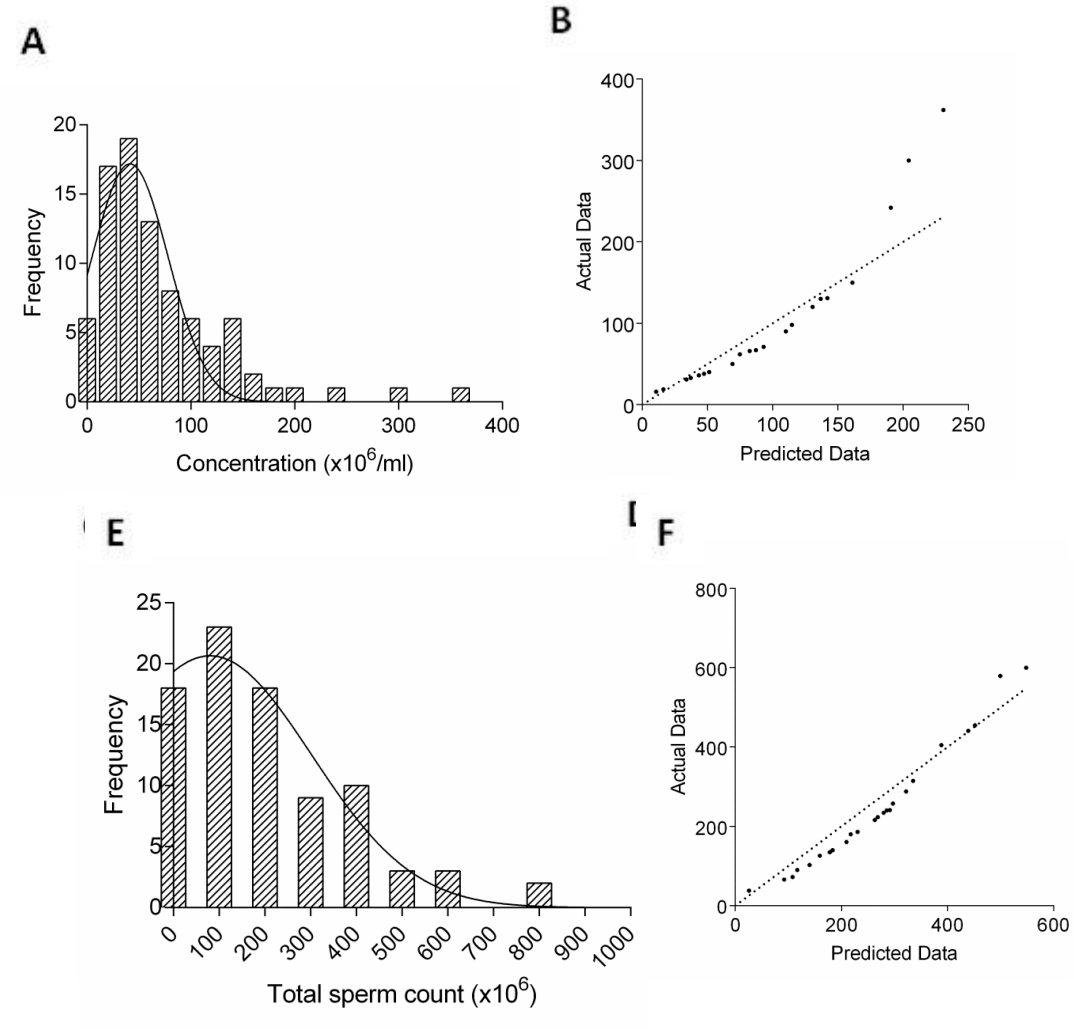
Appendix 6: Normalisation of semen parameter data



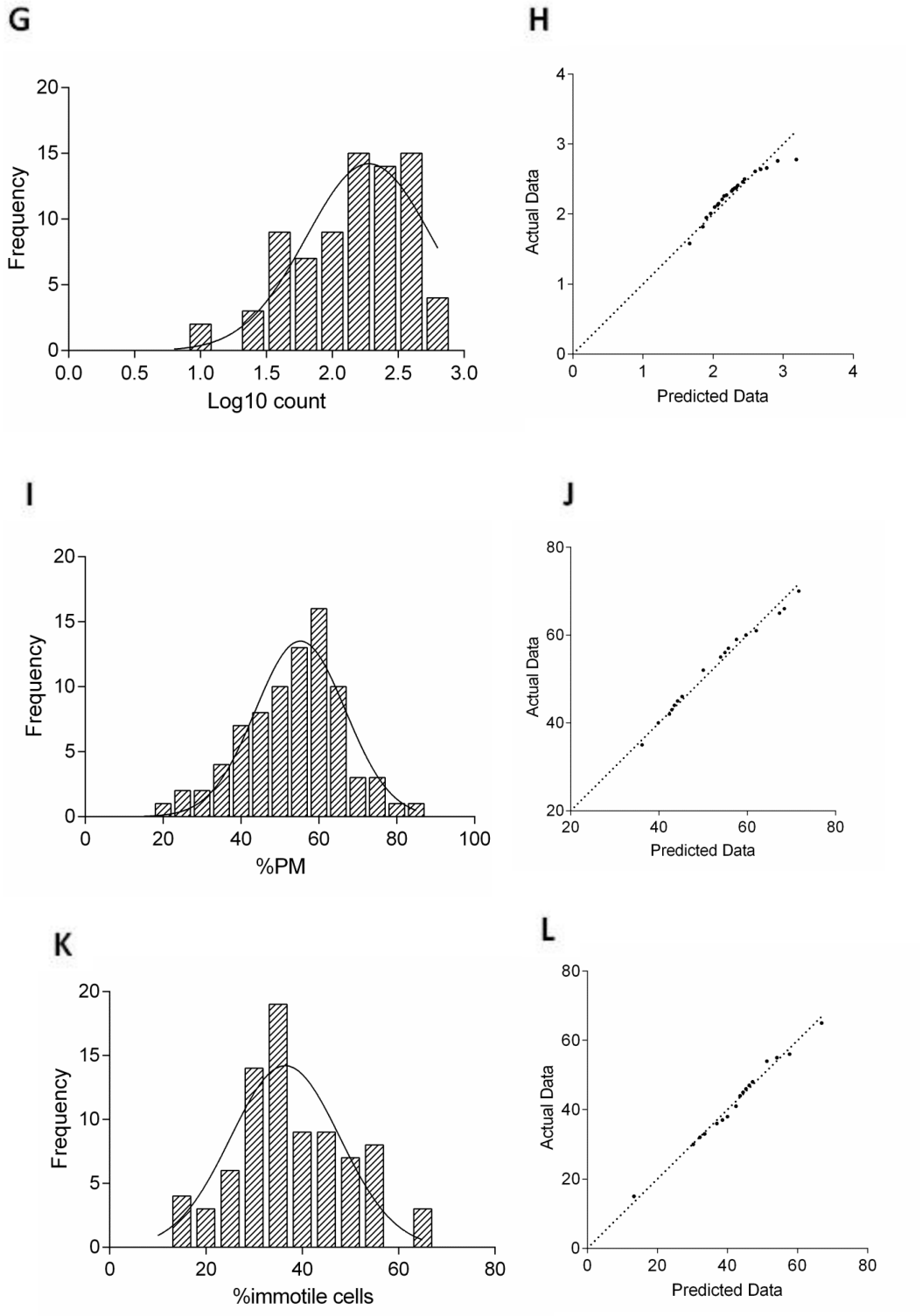


Figure 1. Frequency distribution histograms and Q-Q plots for untransformed and transformed semen parameters.

Sperm concentration and total sperm count were positively skewed (A and E) and did not show the expected linear trend (B and F). Log_{10} transformation made sperm concentration normal (C and D) while sperm count still showed some skewness (G and H). % Progressive motility and % immotile cells showed a normal bell-shaped Gaussian curve (I and K) and fitted the expected normal distribution (J and L), therefore did not require transformation.

Appendix 7: MMU Ethical Approval

FACULTY OF SCIENCE AND ENGINEERING

Manchester Metropolitan University

M E M O R A N D U M

TO Michael Carrol

FROM AnneMarie Walsh

DATE 23rd January 2013

SUBJECT Application for Ethical Approval **(SE111229A01)**

On the 30/07/2013 the Head of Ethics for Science & Engineering considered your application for Ethical Approval (SE111229A01) entitled "Investigating the effect of lifestyle and environment on human sperm". The application has been granted Favourable Opinion and you may now commence the project.

MMU requires that you report any Adverse Event during this study immediately to the Head of Ethics (Prof Bill Gilmore) and the Administrator (AnneMarie Walsh). Adverse Events are adverse reactions to any modality, drug or dietary supplement administered to subjects or any trauma resulting from procedures in the protocol of a study.

An Adverse Event may also be accidental loss of data or loss of sample, particularly human tissue. Loss of human tissue or cells must also be reported to the designated individual for the Human Tissue Authority licence (currently Prof Bill Gilmore).

Regards

AnneMarie Walsh
Research Degrees Group Officer
All Saints North

Page **1** of **1**

Appendix 8: Participant information sheet

Participant information sheet

Title of Study: *Investigating the effect of lifestyle and environment on human sperm.*

Study Background

Male factor infertility can account for 40% of infertility experienced by couples trying to conceive. As sperm is produced continuously in the testis through the process of spermatogenesis – the sperm cells are vulnerable to damage. This damage can occur in the sperm cell membrane and the DNA.

We will investigate how environmental compounds and lifestyle can cause this damage. This information will offer potential therapeutic options that may improve male infertility.

Who can take part?

Any male aged over 18 years old.

What is involved?

You will be required to provide a semen sample either at home or within a secure room at the school of healthcare science. You will produce this semen via masturbation. A full sample is required. You will be provided with a sterile container from which to deposit your specimen.

The semen sample will be assessed for parameters such as motility (how well the sperm are moving), morphology (the shape of the sperm cells) and concentration (the number of sperm per millilitre of seminal fluid). The sperm will then undergo a variety of biochemical and molecular biology tests. The samples will be stored at -80°C for further analysis.

Your sperm sample will NOT, at any time, be used for any assisted reproductive techniques and will ONLY be used for research or teaching purposes.

Please note that this is not a diagnostic test and you will not be informed of the quality of your semen/sperm.

Are there any risks in taking part in the study?

There may be a slight risk of fainting due to the physical activity of semen production. If you are providing the sample on site, the secured room can be locked from the inside. After an allotted time has elapsed, a study team member will knock on the door to ensure you are OK. If there is no answer, they will enter the room using a key to establish your status.

Appendix 9: Medical Screening Questionnaire

Medical Screening Questionnaire

It is important that the investigators are aware of any health conditions before participation in this research study. This information will be kept strictly confidential.

Please answer the following questions as accurately as possible.

Are you currently taking any prescribed medication?	**YES/NO**
Are you currently attending your GP?	**YES/NO**
Have you ever suffered from a cardiovascular problem? i.*e. high blood pressure, anaemia, heart attack etc*	**YES/NO**
Have you ever suffered from a neurological disorder? *i.e. epilepsy, convulsions etc*	**YES/NO**
Have you ever suffered from an endocrine disorder? *i.e. diabetes etc*	**YES/NO**
Do you have Diabetes (Type I or II)	**YES/NO**
Have you ever suffered from a chronic gastrointestinal disorder? *i.e. Crohn's disease, irritable bowel syndrome etc*	**YES/NO**
Have you ever suffered from a skin disorder? *i.e. eczema etc*	**YES/NO**
Do you suffer from any allergies? *i.e. any medications, foods etc*	**YES/NO**
Have you had a vasectomy or any urological surrey? *i.e. testicular surgery*	**YES/NO**
Have you had Mumps?	**YES/NO**
Have you had any testicular injuries / torsions?	**YES/NO**
Do you smoke?	**YES/NO**
Do you knowingly have, or had a Sexually Transmitted Infection?	**YES/NO**

If you have answered "yes" to any of these questions, please provide details below:

Appendix 10: MMU Participant Consent form

Participant informed consent ID code: ……

Name: ……

Date of Birth: …… Age: ……

Investigating the effect of lifestyle and environment on human sperm

Principal Investigator:
Dr Michael Carroll

Investigators/collaborators:
Dr Nessar Ahmed
Dr Christopher Murgatroyd
Prof. Daniel Brison (external collaborator)

Ethics approval number:

I have read and understood the information sheet and all verbal explanation outlining the purpose of the study and the assessments involved.
Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without having to give a reason. I understand that my sperm will not be used, at any time, for any form of assisted reproductive technique, such as fertilising human eggs or the creation of human embryos. I understand that my semen may be also used for teaching purposes. I am happy for blood and saliva to be taken, prepared and stored for research.
My concerns regarding this study have been answered and such further concerns as I have during the time of the study will be responded to. It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the Chair of the Ethics Committee of the School of Healthcare Science, Manchester Metropolitan University, Oxford Road, Manchester M15 5GD.

I give my consent for semen collected from me during the course of this study to be retained in the study biobank at MMU for retrospective biochemical and molecular biology analysis.

Signed _____ Date _____

Name (print) ……..

Appendix 11: Semen Procurement Form

Specimen details

Please complete this form and bring it with you to the laboratory along with your sample and the request form.

Name: _____

Date of Birth: _____

Date of specimen: _____

Time specimen passed: _____

Abstinence (days): _____

I confirm that this semen specimen is mine and I consent to its use for the purpose of research and education.

Signed

_____

Appendix 12: Methylation Data Transformations

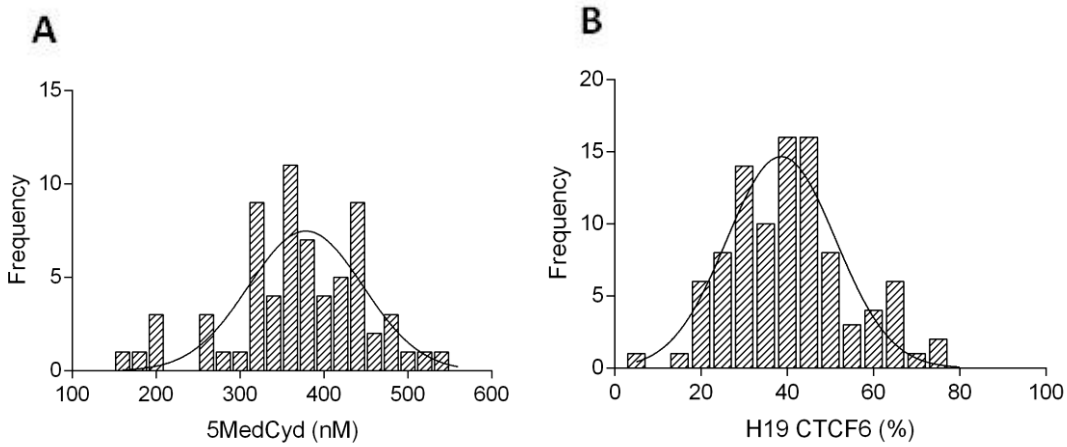


Figure 1

Histograms showing the normal distributions of raw data for global DNA methylation (A) (n=64) and the 6th CTCF binding site of H19 (B) (n=91). Two outliers were excluded from the global methylation dataset (686 µM and 719 µM) as these caused a skew in the data. Once removed the Shapiro-Wilk statistic was 0.968 (*p*=0.098). No outliers were excluded from H19/CTCF6 and the Shapiro-Wilk statistic was 0.985 (*p*=0.406).

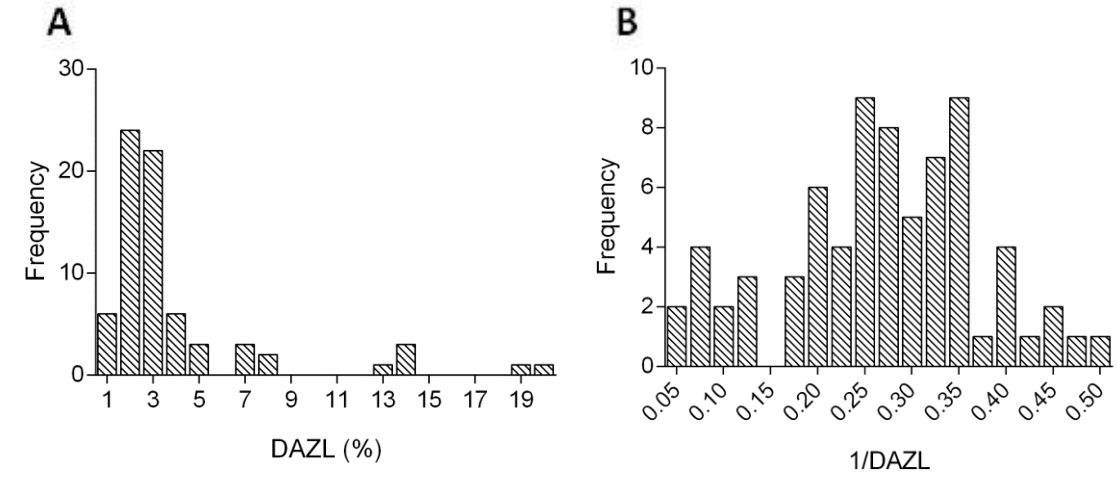


Figure 2

Transformation of DAZL methylation data to a normal distribution using a reciprocal transformation. One extreme lower outlier (0.42%) was excluded from the analysis. Shapiro-Wilk value after transformation was 0.979 (*p*=0.283) showing no skewness in the data.

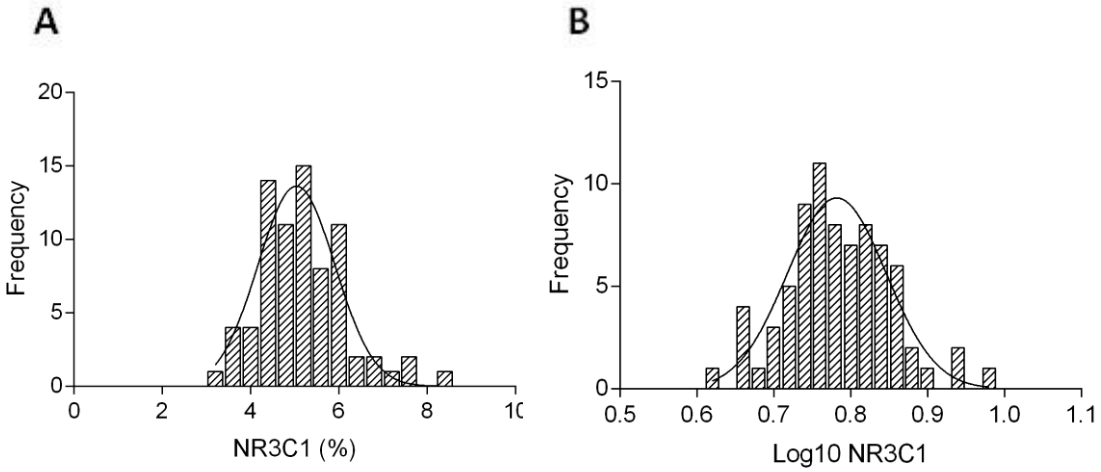


Figure 3

Histograms showing original (A) and log10 transformed (B) hNR3C1 data. The original data was slightly positively skewed (Shapiro-Wilk 0.95, *p*=0.006) while the transformed data showed a distribution closer to a Gaussian curve (Shapiro-Wilk 0.98, *p*=0.286).

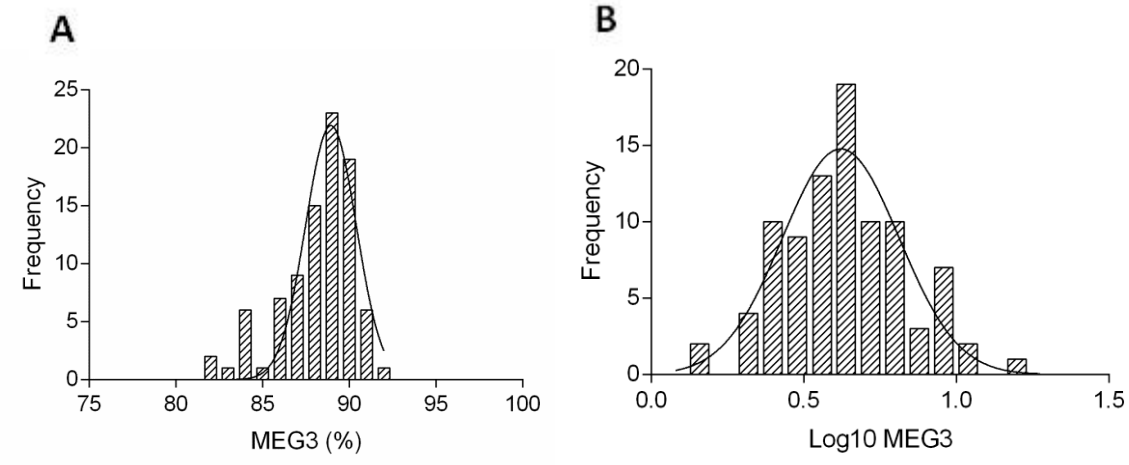


Figure 4

Histograms illustrating the distribution of the MEG3 gene before (A) and after (B) reflection and log10 transformation.

Appendix 13: Data Tables

Table 1 Sperm DNA methylation and semen parameters

Semen parameter	5MedCyd (nM)	H19 CTCF6 %	H19 exon 1 %	PLAGL1 %	SNRPN %	MEG3 %	MTHFR %	DAZL %	NR3C1 %
Concentration (x10^6/ml)									
r	0.127	0.12	0.206	**0.207**	**0.208**	0.156	-0.156	**0.465****	-0.141
p-value	0.311	0.255	0.09	**0.067**	**0.055**	0.146	0.171	**0.000**	0.23
N	66	92	69	**79**	**86**	88	79	**71**	74
%PM									
r	**-0.297***	-0.008	0.151	-0.179	-0.01	**-0.243***	0.038	0.05	0.055
p-value	**0.016**	0.949	0.216	0.115	0.93	**0.023**	0.741	0.676	0.641
n	**66**	92	69	79	86	**88**	79	71	74
%immotile									
r	**0.344****	-0.127	-0.182	**0.214**	0.062	**0.207**	-0.065	-0.016	-0.063
p-value	**0.005**	0.228	0.136	**0.058**	0.569	**0.053**	0.569	0.892	0.595
n	**66**	92	69	**79**	86	**88**	79	71	74
Total count (x10^6)									
r	0.025	0.095	0.168	0.189	**0.215***	0.109	-0.134	**0.337****	-0.183
p-value	0.842	0.374	0.173	0.099	**0.048**	0.322	0.244	**0.005**	0.123
N	64	89	67	77	**85**	85	77	**69**	72

Pearson's correlation, *r*, and significance (*p*) values are shown.

Table 2 Sperm DNA methylation and semen parameters in men with normal sperm

Semen parameter	5MedCyd (nM)	H19 CTCF6 %	H19 exon 1 %	MEG3 %	PLAGL1 %	SNRPN %	MTHFR %	DAZL %	NR3C1 %
Concentration ≥15x10^6/ml									
r	0.083	0.017	-0.105	0.142	0.142	0.069	0.014	**0.348****	-0.009
p-value	0.544	0.882	0.439	0.247	0.247	0.557	0.911	**0.009**	0.949
n	56	77	56	68	68	74	66	**55**	58
%PM ≥32%									
r	-0.211	0.001	0.194	-0.128	-0.172	-0.058	-0.02	-0.05	-0.043
p-value	0.119	0.989	0.145	0.2	0.155	0.619	0.87	0.705	0.742
n	56	80	58	76	70	75	70	59	61
%immotile <60%									
r	**0.286***	0.037	-0.189	0.182	0.141	0.048	-0.002	0.088	0.085
p-value	**0.028**	0.744	0.144	0.11	0.237	0.678	0.988	0.494	0.502
n	**59**	82	61	78	72	77	73	62	64
Total count (x10^6)									
r	-0.066	0.063	-0.197	0.012	-0.059	-0.045	0.084	0.086	-0.108
p-value	0.632	0.593	0.15	0.92	0.636	0.703	0.504	0.534	0.425
n	55	75	55	71	66	73	66	55	57

Pearson's correlation, *r*, and significance (*p*) values are shown.

Table 3 DNA methylation in fertile and infertile men

	5MedCyd	*H19* CTCF6	*H19* DMR	*PLAGL1*	*SNPRN*	*MEG3*	*MTHFR*	*DAZL*	*NR3C1*
Infertile, *n*	43	61	44	53	55	58	54	47	46
Fertile, *n*	22	29	23	26	29	28	25	21	24
P-value	0.772	0.96	0.13	0.618	0.172	0.348	**0.006*** **	0.483	0.903

Independent t-test significance (*p*-values) are shown along with the number of participants in each group. ****p*<0.001.

Table 4 DNA methylation in oligozoospermic men and men with normal sperm

	5MedCyd	*H19* CTCF6 [a]	*H19* DMR [b]	*PLAGL1* [a]	*SNPRN* [b]	*MEG3* [a]	*MTHFR* [a]	*DAZL* [a]	*NR3C1* [a]
Normal, *n*	57	78	57	68	74	74	67	56	59
Oligo, *n*	8	12	10	11	10	12	12	12	11
P-value	0.573	0.204	0.148	0.078	0.173	0.934	0.154	**0.000**** **	0.261

Independent t-test significance (*p*-values) are shown along with the number of participants in each group. Levene's test was used to confirm homogeneity of variance between normal and oligo groups. If Levene's test was not significant ([a]) then results were taken from t-tests assuming equal variance, while if Levene's test was significant ([b]) results were taken from t-tests not assuming equal variance. *****p*<0.0001.

Table 5 DNA methylation and IVF and ICSI outcomes

	5MedCyd	*H19* CTCF6	*H19* exon 1	*MEG3*	*PLAGL1*	*SNRPN*	*MTHFR*	*DAZL*	*NR3C1*
[a]IVF fert. Rate									
p-value	**0.034***	0.948	0.443	0.372	0.922	0.186	0.146	0.995	0.559
n	**36**	51	36	48	47	48	42	36	38
[a]IVF cleavage rate									
p-value	0.357	0.069	0.644	0.863	0.433	0.273	0.411	0.233	0.83
n	36	51	36	48	47	48	42	36	38
[b]IVF pregnancy rate									
p-value	0.089	0.11	0.187	0.605	0.33	0.841	0.552	0.784	**0.027***
n (no/yes)	20/15	27/22	20/15	27/20	25/20	26/20	25/15	25/10	**23/14**
[a]ICSI fert. rate									
p-value	0.461	0.7	0.346	0.442	0.632	0.569	0.762	0.725	0.732
n	27	37	29	36	31	35	35	30	30
[a]ICSI cleavage rate									
p-value	0.901	0.053	0.609	0.333	0.653	0.612	0.541	0.363	0.481
n	25	35	27	30	30	34	33	28	28
[b]ICSI pregnancy rate									
p-value	0.622	0.517	0.941	0.316	0.689	0.078	0.93	0.622	0.482
n (no/yes)	15/6	18/10	16/6	18/9	16/8	17/10	18/8	15/7	16/6

Significant associations were identified using Pearson's correlation between fertilisation and cleavage rates and DNA methylation ([a]), and Mann-Whitney U test for DNA methylation in sperm of pregnant and not pregnant couples ([b]) Tests were run for IVF and ICSI patients separately. Significant correlations are shown in bold. *$p<0.05$.

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